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Genetic deletion and inhibition of the oxygen sensor PHD1 protects against ischemic stroke: a role for neuronal metabolism?

Annelies QUAEGEBEUR

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“You can tell whether a man is clever by his answers.
You can tell whether a man is wise by his questions.”

- Mahfouz Naguib -

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Dankwoord – Words of gratitude

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Annelies

List of abbreviations

α-KG	α-ketoglutarate
α-KGDH	α-ketoglutarate dehydrogenase
αSMA	α- smooth muscle actin
aCSF	Artificial CSF
ADP	Adenosine diphosphate
ALS	Amyotrophic Lateral Sclerosis
AMP	Adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANLS	Astrocytic-neuronal lactate shuttle
APC/C-Cdh1	Anaphase-promoting complex/cyclosome
ASO	Antisense oligonucleotides
ATF4	Activation transcription factor 4
ATP	Adenylate triphosphate
BBB	Blood-brain barrier
Bdnf	Brain-derived neurotrophic factor
BNIP3	Bcl-2/adenovirus E1B 19-kDa-interacting protein 3
C57BL/6	C57 black 6
CAMKII	Ca2+/calmodulin-dependent protein kinase
CM-H_2-DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CoA	Co-enzyme A
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DIV	Day *in vitro*
DMEM	Dulbeco's Modified Eagle's Medium
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol

E3	E3 ubiquitin ligase
EAAT	Excitatory amino acid transporters
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
ETC	Electron transport chain
FIH	Factor inhibiting HIF
FITC	Fluorescein isothiocyanate
FOXO3	Forkhead box O3
fructose-1,6-P	Fructose-1,6-bisphosphate
fructose-6-P	Fructose-6-phosphate
G6PD	Glucose-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCLC	Glutamate—cysteine ligase catalytic subunit
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
Glc-6-P	Glucose-6-phosphate
Gln	Glutamine
GLS	Glutaminases
Glu	Glutamate
Glud	Glutamate dehydrogenase
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Monomeric glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HClO4	Perchloric acid
HIF	Hypoxia-inducible factor
Hk1	Hexokinase

HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine Phosphoribosyltransferase 1
HRE	Hypoxia-responsive element
IDH2	Isocitrate dehydrogenase 2
KOH	Potassium hydroxide
LDH	Lactate dehydrogenase
MALAT-1	Metastasis associated lung adenocarcinoma transcript 1
$MAP2^{+}$	Microtubule associated protein-2 positive cells
MCT	Monocarboxylate transporters
ME	Malic enzyme
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
NG2	Neural/glial antigen 2
Ngf	Nerve growth factor
NHE-1	Na^{+}/H^{+} exchanger 1
NMDA	N-methyl-D-aspartate
NNT	Nicotamide nucleotide transhydrogenase
NO	Nitric oxide
Nt3	Neurotrophin 3
$O_2^{\cdot -}$	Superoxide radical
OCR	Oxygen consumption rate
OCR_{BAS}	Baseline OCR
OCR_{MAX}	Maximal OCR
OCT	Optimal cutting temperature
$OH^{\cdot}$	Hydroxyl radicals
Olig2	Oligodendrocyte transcription factor
OND	Oxygen-nutrient deprivation
$ONOO^{-}$	Peroxynitrite

OXPHOS	Oxidative phosphorylation
oxPPP	Oxidative pentose phosphate pathway
p75	P75 neurotrophin receptor
PAS	Periodic Acid Schaff
PDK	Pyruvate dehydrogenase kinase
PFA	Paraformaldehyde
PFK-1	Phosphofructokinase 1
PFKFB3	Phosphofructokinase-2,6-fructosebiphosphatase
Pgam2	Phosphoglycerate mutase 2
PHD	Prolyl hydroxylase domain protein
PKM2	M2 isoform of pyruvate kinase
pMCAO	Permanent middle cerebral artery occlusion
PPP	Pentose phosphate pathway
Prdx1	Peroxiredoxin 1
PU4ii	Polyurethane resin
PUMA	P53-upregulated mediator of apoptosis
qPCR	Quantitative polymerase chain reaction
ribose-5-P	Ribose-5-phosphate
ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13a
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Sesn1	Sestrin 1
shRNA	Short hairpin ribonucleic acid
SOD2	Superoxide dismutase 2
t-PA	Tissue plasminogen activator
TCA	Tricarboxylic acid cycle
tMCAO	Transient middle cerebral artery occlusion
TOMM20	Translocase of outer mitochondrial membrane 20 homolog

TrkB	Tyrosine receptor kinase B
TrkC	Tyrosine receptor kinase C
TTC	Triphenyltetrazolium hydrochloride
VEGF	Vascular endothelial growth factor
Vegfr2	Vascular endothelial growth factor 2
VHL	Von Hippel Lindau
WT	Wild type

Chapter I

LITERATURE STUDY

Partially published in Quaegebeur A and Carmeliet P. Oxygen sensing: a common crossroad in cancer and neurodegeneration. Curr Topics Microbiol Immunol 345, 71-103 (2010).

1 INTRODUCTION: AND THEN THERE WAS OXYGEN…

Since the appearance of oxygen in the atmosphere more than 2 billion years ago, eukaryotic life has become critically dependent on aerobic metabolism (Semenza 2007). Since then, molecular oxygen (O_2) serves as terminal electron acceptor in a process referred to as oxidative phosphorylation, providing the cell with a highly efficient means of energy production. However, given the reactive nature of oxygen, mitochondrial oxidative metabolism also exposes the cell to the threat of reactive oxygen species (ROS). Both decreased oxygen levels (hypoxia) and oxidative stress, resulting from excessive ROS generation, posed some of the greatest evolutionary challenges for aerobic life (Taylor and Pouyssegur 2007). Therefore, it is not surprising that organisms have developed a variety of oxygen sensing and adaptive systems, allowing a tightly regulated oxygen homeostasis.

One of these oxygen sensing mechanisms is provided by the family of the prolyl hydroxylase domain proteins. In conditions of low oxygen availability, they will activate various protective responses that serve to restore the oxygen homeostasis. This evolutionary conserved adaptive program has received great medical attention in recent years as hypoxia-related stress and deregulated oxygen homeostasis are commonly implicated in acute insults (e.g. ischemia of organs) as well as chronic disease states

(e.g. cancer, neurodegeneration and inflammation) (Quaegebeur and Carmeliet 2010).

During this doctoral thesis, I focused on the role of these prolyl hydroxylase domain proteins in brain ischemia with specific attention to the question whether and how effects on neuronal metabolism could confer neuroprotection. As an introduction to the data obtained during this doctoral work, I will first give an overview of the described functions of the PHDs. Next, I will review why the brain is so vulnerable to ischemia, discussing the knowns and the unknowns of brain metabolism. Then, I will introduce ischemic stroke and neurodegenerative disorders as two examples of disturbed oxygen homeostasis. Finally, I will elaborate on the hypothesis that inhibition of PHDs would be beneficial in these brain diseases.

2 PROLYL HYDROXYLASE DOMAIN PROTEINS: A FAMILY OF OXYGEN SENSORS

2.1 The Physiology of Oxygen Sensing

For aerobic energy generation, cells are dependent on a regular oxygen supply. Vitally important for maintaining metabolic homeostasis in a cell is that oxygen consumption and delivery are in balance. However, discrepancies between oxygen delivery and demand are common, not only in diverse disease states (e.g. ischemia and cancer). Also in physiological conditions, cells are often exposed to fluctuations in oxygen levels (e.g. upon physical exercise). When oxygen consumption exceeds supply, cellular oxygen levels will drop and hypoxia arises. This will abruptly threaten the energetic balance, resulting in potentially life-threatening damage of an organ or tissue. Aerobic species have therefore developed various oxygen sensing mechanisms during

evolution, both at the cellular and systemic level, as a protection against these conditions of insufficient oxygen supply (Quaegebeur and Carmeliet 2010).

At the systemic level, chemoreceptors in the carotid and aortic bodies signal changes in oxygen, CO_2 and pH levels to cardiorespiratory centers in the central nervous system (Taylor et al 1999). These will adapt respiration, heart rate and blood pressure to ensure adequate oxygenation of the blood and sufficient perfusion. Another homeostatic mechanism acting at the systemic level is the production of erythropoietin (EPO) in the kidney, triggered by systemic hypoxia. EPO will stimulate erythrocyte production and in this way enhance the oxygen carrying capacities of the blood (Jelkmann 2011).

Oxygen sensing at the cellular level is mainly orchestrated by the prolyl hydroxylase domain proteins (Kaelin and Ratcliffe 2008), which were the focus of this doctoral thesis. This family of prolyl hydroxylase domain proteins activate in an oxygen-dependent manner a major transcriptional pathway governed by the hypoxia-inducible factors, allowing the cell to transduce oxygen levels to adaptive gene expression.

2.2 Prolyl Hydroxylase Domain proteins: the molecular players of cellular oxygen sensing

The Prolyl Hydroxylase Domain proteins, or shortly PHDs, belong to a family of 2-oxoglutarate dependent iron(ii)-dioxygenases. PHDs are enzymes that hydroxylate different target proteins at specific proline residues. PHDs act as *bona fide* oxygen sensors because they use molecular oxygen as a substrate in their hydroxylation reaction (Aragones et al 2009, Kaelin and Ratcliffe 2008, Quaegebeur and Carmeliet 2010, Schneider et al 2009). Mammalian cells possess three different PHD isoforms (PHD1-3).

The best-known hydroxylation targets of the PHDs are the hypoxia-inducible factors (HIFs). This transcription factor family acts as a master regulator of oxygen homeostasis by engaging an adaptive transcriptional

response. More than 20 years ago, EPO was discovered as the first gene regulated by HIF (Semenza and Wang 1992). To date, this HIF family is believed to control the expression of possibly more than 1000 genes (Semenza 2014). HIF is a heterodimer, composed of an oxygen-labile HIF-α subunit and constitutive HIF-β subunit. In mammals, HIF-α exists as 3 isoforms (HIF-1α, -2α and -3α) of which HIF-1α and HIF-2α are best characterized (Majmundar et al 2010).

When oxygen is sufficiently present, PHDs hydroxylate two proline residues of HIF-α (pro402 and pro564) located on the oxygen-degradation domain of HIF-α. The Von Hippel Lindau (VHL) protein will recognize this posttranslational modification and will recruit an E3 ubiquitin ligase complex that via ubiquitination will target HIF-α for proteasomal degradation (Kaelin and Ratcliffe 2008, Schofield and Ratcliffe 2004). In other words, in normoxic conditions, PHDs' hydroxylation activity keeps HIF-α levels low (Figure 1). Indeed, in normoxia, HIF-α has a half-life of less than 5 minutes (Berra et al 2001, Huang et al 1998). Consequently, when oxygen levels drop, PHDs lose their activity and HIF-α will no longer be degraded. The accumulating HIF-α will form a complex with its constitutively expressed counterpart HIF-β (also known as the aryl hydrocarbon receptor nuclear translocator) and bind to the hypoxia-responsive element (HRE) in the promotor of numerous genes (Figure 1). This transcriptional response can affect a broad range of biological processes but most importantly, it will affect the vasculature and cellular metabolism. By increasing the expression of angiogenic factors such as vascular endothelial growth factor (VEGF), oxygen supply will be promoted, while cellular metabolism will be adapted to reduced oxygen levels, for instance by increasing the expression of pyruvate dehydrogenase kinase (PDK), thereby inhibiting the mitochondrial respiration (Quaegebeur and Carmeliet 2010). In the next paragraph, I will elaborate further on how PHDs regulate this metabolic homeostasis and more specifically, how their biological and catalytic properties allow them to both sense and instruct changes in vascular supply and cellular metabolism.

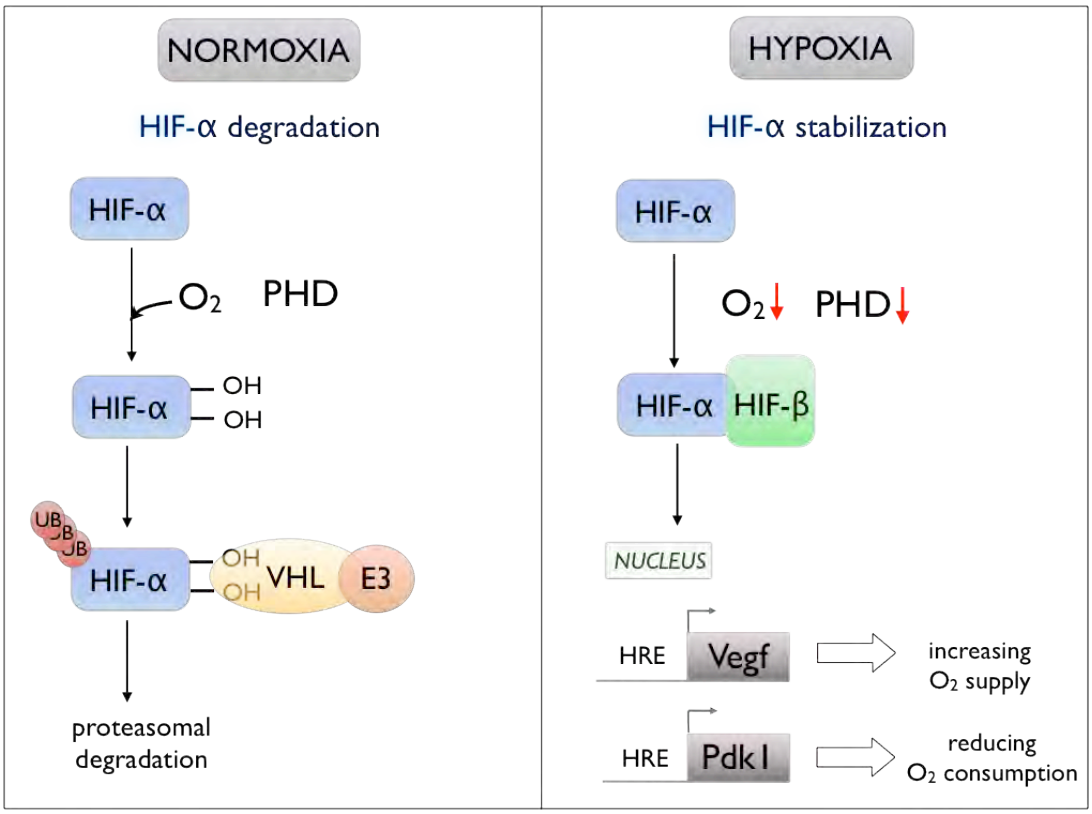

Figure 1: PHDs regulate HIF-α stability in an oxygen-dependent manner

In normoxia, PHDs will hydroxylate HIF-α. This hydroxylation reaction will lead to the recruitment of Von-Hippel-Lindau (VHL) and E3 ubiquitin ligase (E3) that via ubiquitination will target HIF-α for proteasomal degradation. In hypoxia, PHDs lose their activity, allowing the HIF-α subunit to dimerize with the constitutive HIF-β subunit. Via binding to the hypoxia-responsive element (HRE), this complex of transcription factors will stimulate gene expression with the purpose of restoring the oxygen homeostasis. Vegf: vascular endothelial growth factor; Pdk1: pyruvate dehydrogenase kinase 1.

2.3 Vessels and metabolism: the IN- and OUTPUT of PHDs

The nature of stimuli to which PHDs respond and the biological processes regulated by PHDs, make PHDs the paramount orchestrators of a tight oxygen and metabolic homeostasis. Indeed, they feed back to processes that influenced their activity in the first place.

2.3.1. INPUT – TO WHICH FACTORS DOES PHD ACTIVITY RESPOND?

As outlined earlier, oxygen levels dictate PHD activity. However, not only oxygen, but also 2-oxoglutarate is required for the proline-hydroxylation function, as well as a ferrous iron molecule (Fe^{2+}) in the catalytic core of the PHDs (Quaegebeur and Carmeliet 2010). Therefore, PHDs have a function that goes beyond oxygen sensing *strictu sensu,* as they are sensitive to various metabolic signals (Figure 2). Hence, the catalytic properties of the PHDs are most elegantly designed to allow them orchestrating this complex metabolic equilibrium.

a. *Oxygen*

PHD activity responds to oxygen levels, as molecular oxygen is required as a substrate in the hydroxylation reaction. Hypoxia arises when the oxygen supply does not meet the oxygen demand and results in a drop in ATP production. This occurs during embryogenesis (when the embryo becomes too large to rely on simple oxygen diffusion) or in the context of various diseases such as infarctions (when an occluded blood vessel acutely deprives tissue from oxygenation) and cancers (when malignant cells outgrow their own vasculature). The rather low affinity of PHDs to oxygen (K_m of 100-250 mM) allows PHDs to act dynamically in the physiological oxygenation range (Ehrismann et al 2007, Koivunen et al 2004). This implies that small alterations in oxygenation will result in substantial effects on PHD activity and that PHDs will already initiate an

adaptive response before oxygen tension will drop too far to maintain mitochondrial respiration.

b. *Tricarboxylic acid (TCA) cycle metabolites*

Besides oxygen, the levels of TCA cycle metabolite 2-oxoglutarate (α-ketoglutarate) will also affect PHDs' activity because α-ketoglutarate undergoes oxidative decarboxylation to succinate during the hydroxylation reaction (Epstein et al 2001). This mechanism most likely explains why drugs showing structural analogy with 2-oxoglutarate (e.g. dimethyloxalylglycine (DMOG)) as well as amino acid starvation inhibit PHD function due to competitive inhibition with and depletion of 2-oxoglutarate respectively (Duran et al 2013, Fraisl et al 2009). This metabolic regulation is also exemplified by the PHD-HIF pathway activation in malignant tumors based on loss-of-function mutations in fumarate dehydrogenase and succinate dehydrogenase (Isaacs et al 2005, Selak et al 2005). The accumulation of fumarate and succinate respectively, is believed to inhibit PHDs via product inhibition. Conversely, the R-enantiomer of oncometabolite 2-hydroxyglutarate, accumulating in gliomas due to isocitrate dehydrogenase (IDH) mutations, was recently shown to activate PHDs (Koivunen et al 2012).

c. *Redox state*

As shown in figure 2, PHD activity also requires iron in the catalytic site for its activity. This iron has to be reduced (Fe^{2+}) in order to enable hydroxylation activity. This suggests that PHD activity would be sensitive to the redox state of a cell, since an excess of oxygen radicals would oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) (Gerald et al 2004). Treatment of cells with anti-oxidants (e.g. ascorbate) indeed prevents HIF stabilization (Gerald et al 2004, Knowles et al 2003). However, it remains controversial to what extent redox state affects PHD activity in non-hypoxic conditions. Of note, this mechanism also implies that iron

depletion (e.g. by iron chelating agents) will result in HIF accumulation via PHD inhibition (Epstein et al 2001).

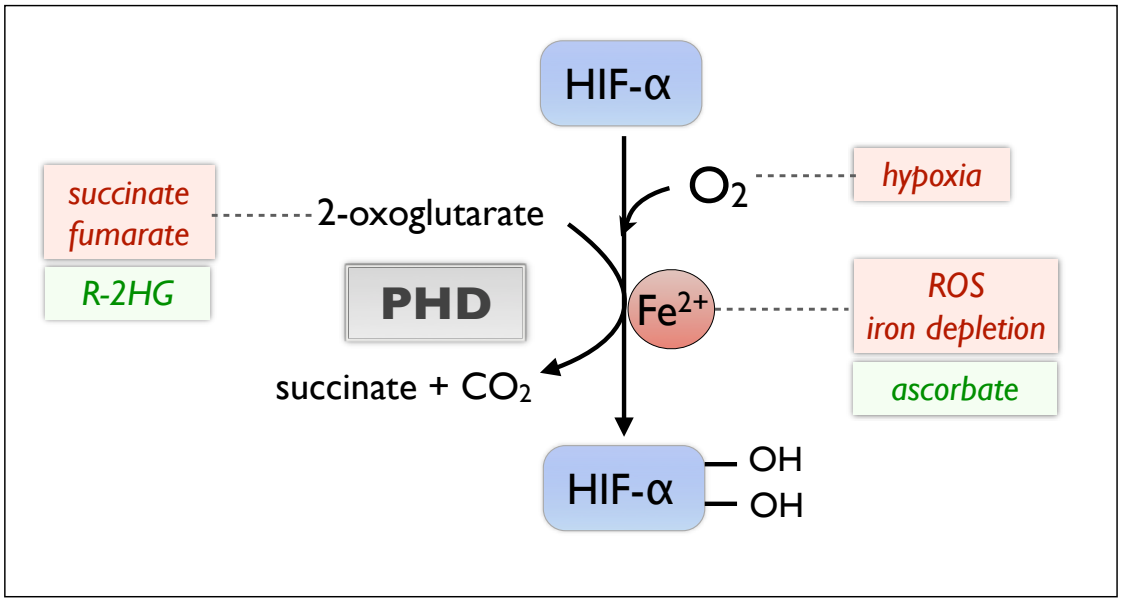

Figure 2: PHDs as paramount cellular integrators of different metabolic signals

PHDs do not only need oxygen to carry out the hydroxylation reaction, both 2-oxoglutarate and iron (Fe^{2+}) are also used as cofactors. In red are the signals that will temper PHD activity. In green are the signals that will promote PHD activity. ROS: reactive oxygen species; R-2HG: R-enantiomer of 2-hydroxyglutarate.

2.3.2. OUTPUT: VASCULAR AND METABOLIC EFFECTS

PHDs do not merely function as sensors of insufficient oxygen supply or metabolic imbalance. They will directly instruct adaptations in an attempt to re-establish the oxygen homeostasis. This hypoxic response involves a broad variety of biological processes – beyond vessels and metabolism – such as inflammation, autophagy, endoplasmatic reticulum stress, apoptosis, cell stem physiology etc. (Quaegebeur and Carmeliet 2010). With oxygen delivery and consumption as ultimate determinants of the hypoxia response, I will limit further discussion to the metabolic and vascular effects mediated by PHD

inhibition. Essentially, PHD signaling will initiate programs that enhance oxygen supply via “cell extrinsic” mechanisms on the one hand, and that reduce energy expenditure and oxygen consumption via “cell intrinsic” mechanisms on the other hand (Figure 3).

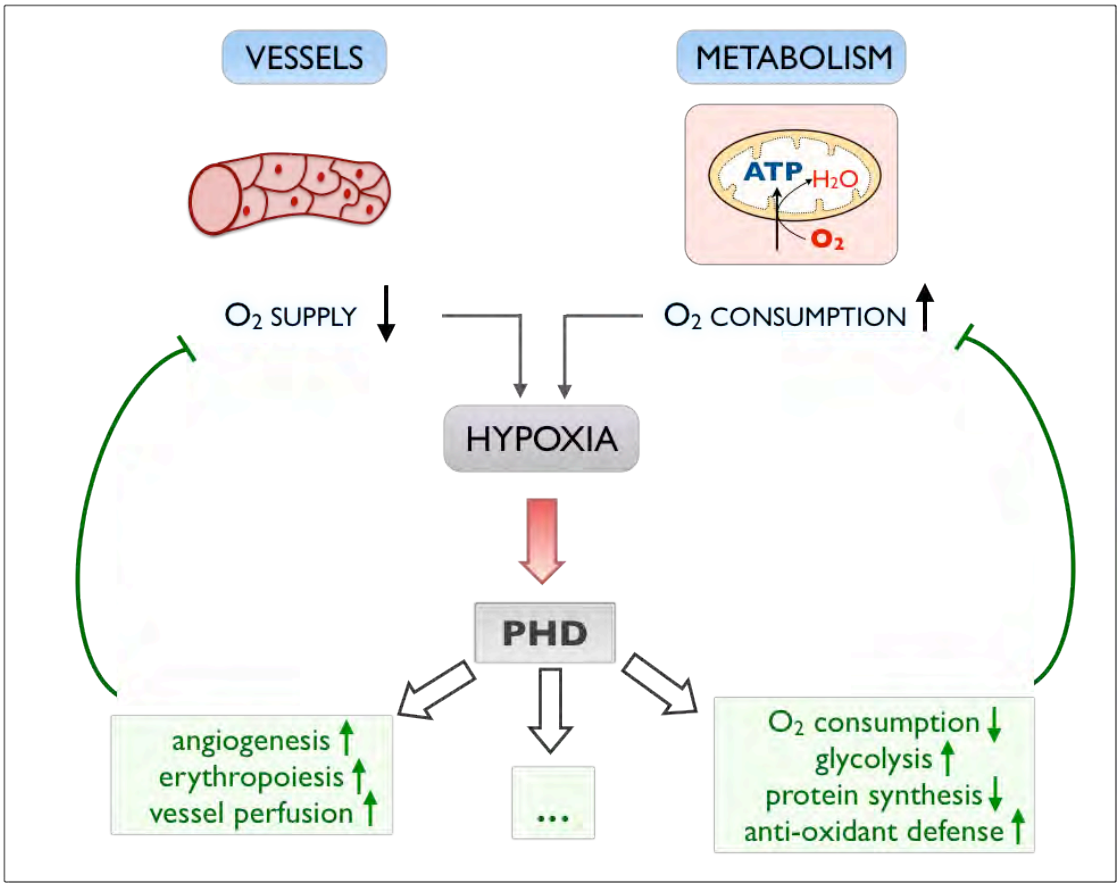

Figure 3: **Vessels and metabolism: the messengers to and the actors of the PHDs**

Scheme on the main functions of the PHDs showing that, in hypoxia, reduced PHD activity will activate a variety of responses in order to safeguard the oxygen homeostasis. At the vascular level, angiogenesis, erythropoiesis and vessel perfusion will be promoted. Metabolic changes aim to enhance the cell’s resilience against hypoxia and ROS by decreasing its oxygen consumption, increasing glycolysis, reducing protein synthesis and promoting anti-oxidant activity. This scheme is a simplification as besides vessels and metabolism many other cellular processes (…) can be affected by PHDs.

a. EXTRINSIC mechanisms – OXYGEN SUPPLY

As organisms grew larger over time in evolution, more complex and elaborate transport systems for oxygen delivery arose. During embryogenesis, the circulation is the first system to become functional (Semenza 2014). In adult organisms, the vascular network is dormant in health, but when a tissue is deprived of oxygen, it will induce a response designed to increase oxygen delivery by enhancing angiogenesis and erythropoiesis. Genetic mouse studies demonstrated that PHDs participate in this adaptive response.

i. erythropoiesis

A role in erythropoiesis has been reported for several PHD isoforms, but most clearly for PHD2. Postnatal PHD2 deletion (Minamishima et al 2008, Minamishima et al 2009, Takeda et al 2008) as well as combined loss of PHD1 and PHD3 (Takeda et al 2008) result in polycythemia due to increased renal EPO production. The discovery of heterozygous mutations in the *PHD2* gene in patients with idiopathic erythrocytosis underscores the relevance of PHDs in erythropoiesis in humans (Percy et al 2006). In addition, combined loss of PHD1, PHD3 and hepatic PHD2 reactivates hepatic EPO production (Minamishima and Kaelin 2010). Not surprisingly, clinical trials with PHD inhibitors are currently running to treat anemia due to kidney failure.

ii. angiogenesis

Effects on angiogenesis seem so far to be exclusively linked to PHD2. Postnatal disruption of PHD2 increases blood vessels in different organs (Takeda et al 2007) resulting in dilated cardiomyopathy and circulatory congestion (Minamishima et al 2008). The effects of PHD2 deficiency on the vasculature seem to be dependent on the residual PHD2 activity and the context. Indeed, heterozygous deletion of PHD2 was shown to normalize tumor vessels, thereby improving perfusion and chemotherapy delivery to the tumor (Leite de Oliveira et al 2012, Mazzone et al 2009), as well as to

stimulate collateral artery growth, governing protection against limb ischemia (Takeda et al 2011). Interestingly, the downstream effectors of PHD2 can vary depending on the organ and context. Whereas partial PHD2 deficiency in endothelial cells instructed tumor vessel normalization via HIF-2α (Mazzone et al 2009), the angiogenic response in postnatally PHD2-deficient mice was more likely to be HIF-1α-dependent (Takeda et al 2007) and the increased arteriogenesis in limbs of heterozygous PHD2 deficient mice was HIF-independent (see below) (Takeda et al 2011).

b. INTRINSIC mechanisms – CELLULAR METABOLISM

i. Introduction on cellular metabolism

Cellular metabolism can be defined as the set of chemical reactions within a cell. One of the main functions of cellular metabolism is to generate energy that cells use to fuel various processes such as protein synthesis, organelle turnover, ion homeostasis and many others. In almost any mammalian cell type, glucose is a major source for ATP production. Upon entering the cell, glucose will be converted into pyruvate in a process that is referred to as "glycolysis". Essentially, pyruvate has two major fates. Pyruvate can be metabolized to lactate, which will be transported outside of the cell. This process is known as "anaerobic glycolysis" or "fermentation" and generates 2 molecules of ATP per molecule of glucose.

Alternatively, pyruvate will enter the mitochondria and via different oxidation steps in the TCA cycle give rise to nicotinamide adenine dinucleotide (NADH) molecules, which serve as electron donor for the electron transport chain (ETC). Here, electrons are passed along a chain of redox complexes, enabling a proton gradient to be built up across the mitochondrial membrane, necessary for ATP production. Oxygen is the final electron acceptor at the end of the ETC. Oxidation of one glucose molecule yields 36 molecules of ATP, which leaves this process of "oxidative phosphorylation"

the more efficient way of generating energy in normoxic conditions (Figure 4) (Berg 2007). As an obligatory byproduct of oxidative phosphorylation, superoxide radicals will be generated when electrons escape the ETC prematurely and react with oxygen molecules.

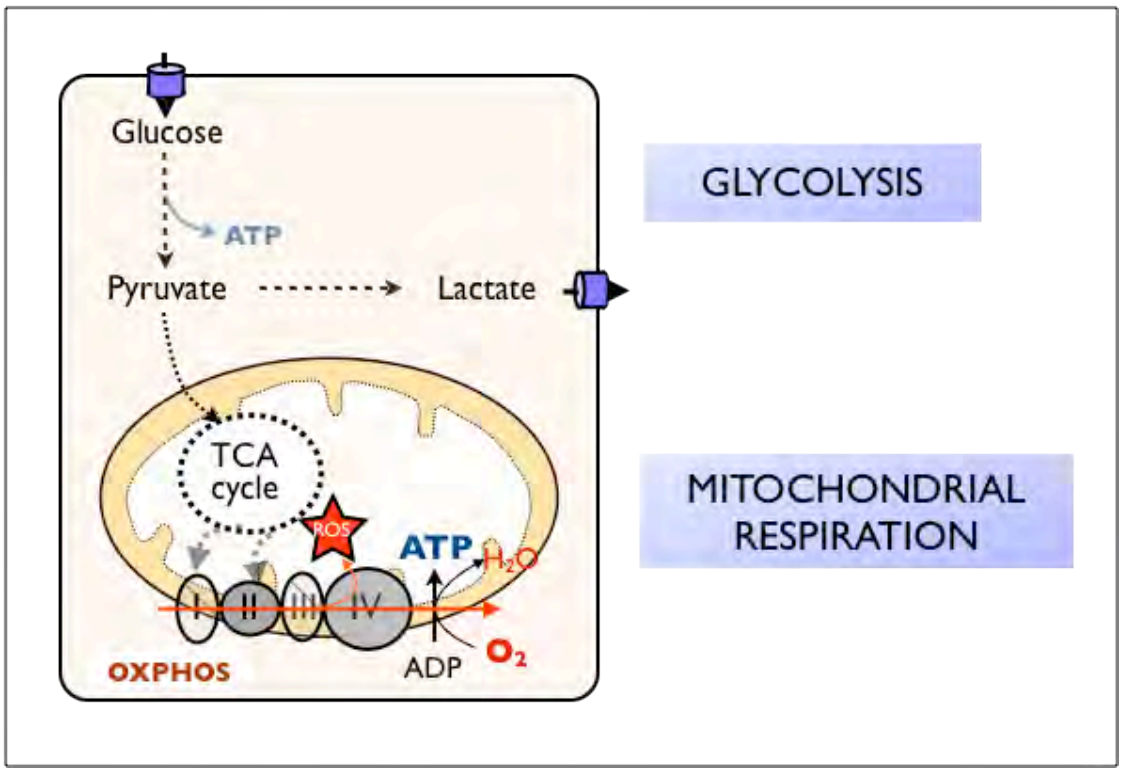

Figure 4: The basics of cellular glucose metabolism.

Glycolysis refers to the stepwise conversion of glucose into pyruvate. Pyruvate can either be converted to lactate or enter the mitochondria where it undergoes different oxidation steps in the TCA cycle. Electron transfer along the complexes of the electron transport chain will result in ATP production and O_2 consumption. As a normal byproduct of this process of oxidative phosphorylation (OXPHOS), limited amounts of superoxide radicals (ROS) will be generated. Note: glycolysis generates low amounts of ATP, but allows energy production in hypoxia, whereas the process of mitochondrial respiration is a more efficient way of energy generation. For reasons of simplicity and clarity, the stoichiometry of the reactions has been omitted.

ii. The role of HIF-α in hypoxic metabolism

When oxygen supply becomes limiting, cellular oxygen levels will drop to a level where the electron transport through the ETC (and consequently, ATP production) can no longer meet the ATP demand. This hypoxic event will have two major metabolic consequences: first, the acute drop in ATP production will lead to an energetic crisis, jeopardizing all the energy-consuming cellular processes. Second, and somewhat counter intuitively, hypoxia results in a surge in mitochondrial ROS generation (Chandel et al 2000). Increased $NADH/NAD^+$ ratio and reduction of electron carriers likely favor electron leakage and generation of superoxide radicals. Especially at complex I and III, electrons can escape and react with the limited oxygen molecules still present, generating excessive amounts of reactive oxygen species (ROS), threatening the structural and functional integrity of the cell (Bolanos et al 2009).

As a protective physiological response to this hypoxic stress, a cell will generally switch from aerobic metabolism to anaerobic glycolysis, an oxygen-independent way of energy generation. In various papers, this hypoxia-induced metabolic rewiring has been linked to HIF-1α activation (Schneider et al 2009). HIF-1α accumulation in hypoxia will regulate the following metabolic processes in order to protect the cell against metabolic demise (Figure 5):

1) Reducing oxygen consumption (energy conservation)
   HIF-1α will shut down mitochondrial respiration in order to adjust the oxygen consumption to the lower oxygen levels. By inducing pyruvate dehydrogenase kinase (PDK), which inhibits pyruvate dehydrogenase, HIF-1α will prevent the entry of pyruvate into the mitochondria (Kim et al 2006, Papandreou et al 2006). Another way of HIF-1α lowering mitochondrial respiration is by downregulating the expression of succinate dehydrogenase subunit B (SDH-B) (Dahia et al 2005). HIF-1α will also lower mitochondrial content by stimulating mitophagy and

counteracting mitochondrial biogenesis (Zhang et al 2007, Zhang et al 2008). At the same time, the residual respiration will become more efficient by a HIF-mediated switch from isoform Cox4.1 to Cox4.2 (Fukuda et al 2007), where the latter has an improved affinity for oxygen.

2) Increase of anaerobic glycolysis (energy compensation)

In an attempt to generate sufficient ATP despite hypoxia, HIF-1α will promote the glycolytic flux. Hereto HIF-1α will upregulate the expression of several glycolytic genes: glucose transporter GLUT1, hexokinase, enolase, lactate dehydrogenase (LDH) etc. (Denko 2008). More recently, the M2 isoform of pyruvate kinase (PKM2), catalyzing the last step in glycolysis, was identified as a novel target of HIF-1α in cancer cells (Luo et al 2011). PKM2 is mainly expressed in proliferating cells and is less active than the PKM1 isoform (Vander Heiden et al 2010). It is believed that via accumulating glycolytic intermediates, PKM2 expression in cancer cells facilitates the generation of macromolecular precursors, necessary for biomass duplication.

To prevent intracellular acidification, the accumulating lactate in the cell will undergo efflux via monocarboxylate transporters (MCTs) and the Na^+/H^+ exchanger 1 (NHE-1). The expression of these transporters is also driven by HIF-1α (Shimoda et al 2006, Ullah et al 2006). Hypoxia will also indirectly enable anaerobic glycolysis by promoting glycogen accumulation and utilization. Indeed, both glycogen synthase and glycogen phosphorylase, the enzymes responsible for glycogen synthesis and glycogen breakdown respectively, are induced in hypoxic conditions (Favaro et al 2012).

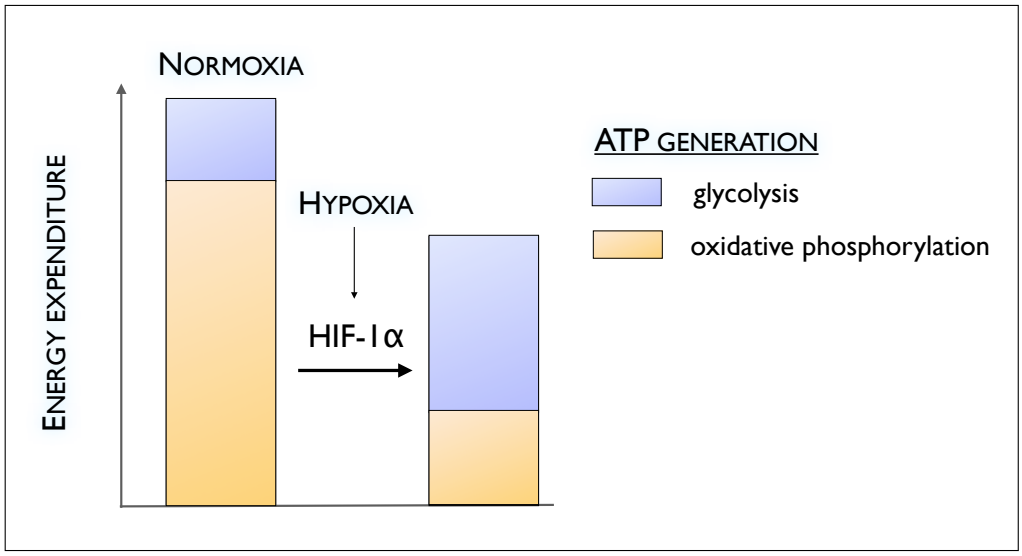

Figure 5: **The canonical role of HIF-1α in hypoxic metabolism**.

In normoxia, most cells will generate ATP molecules predominantly via mitochondrial metabolism (orange), as this yields 15 fold more ATP molecules per molecule of glucose than glycolysis. In hypoxia, HIF-1α will a) lower mitochondrial respiration; b) increase glycolytic ATP production; and c) reduce energy expenditure.

3) Reducing energy expenditure (oxygen conformance).

   In order to equalize energy need to the reduced energy generating potential, HIFs will shut down non-essential processes. For instance, HIF-1α will inhibit overall protein synthesis via inhibition of the mammalian target of rapamycin (mTOR) pathway (Brugarolas et al 2004). Conversely, HIF is also able to stimulate biosynthesis especially in cells that continue their proliferation in hypoxia such as cancer cells. HIF-1α was found to stimulate the flux of glycolytic intermediates through the non-oxidative branch of the pentose-phosphate pathway, thereby generating ribose-5-phosphate, which is used as a precursor for nucleotide synthesis (Zhao et al 2010). Additionally, HIF-1α was linked to reductive metabolism of glutamine in order to maintain fatty acid synthesis (Gameiro et al 2013, Metallo et al 2012, Mullen et al 2012). Lipid synthesis requires acetyl-CoA,

which becomes limiting in hypoxic conditions given the reduced entry of pyruvate into the mitochondria. Via stimulating the reductive carboxylation of α-ketoglutarate (originating from glutamine) to citrate, HIF-1α replenishes the acetyl-CoA pools. Different mechanisms have been proposed: in one study, HIF-1α was found to impair the activity of α-ketoglutarate dehydrogenase (α-KGDH) via Siah2-mediated degradation of one of the subunits of α-KGDH (Sun et al 2014). Another study showed the necessity of ongoing α-KG oxidation in order to generate reducing equivalents for the reductive carboxylation (Mullen et al 2014).

Many of these alterations have been shown to be HIF-1α-mediated, even though an additional role for HIF-2α is plausible. Nevertheless, it seems that HIF-1α and HIF-2α regulate rather distinct sets of metabolic genes. The role of HIF-2α in metabolism needs therefore further investigation (Majmundar et al 2010). The common interpretation of these HIF-mediated metabolic alterations is that they enable anaerobic energy generation. However, at O_2 levels of 1%, the oxygen tension in the mitochondria is not limiting for ATP production, triggering the question whether this drop in mitochondrial respiration would not serve redox rather than bioenergetics purposes by limiting the mitochondrial production of ROS (Semenza 2012).

iii. The role of PHDs in hypoxic metabolism

1) PHD1

Genetic studies in PHD deficient mice have shown that PHDs are involved in the metabolic switch from oxidative to glycolytic metabolism and hence act as key regulators of cellular metabolism. PHD1 is the isoform that has been most extensively linked to this hypoxic-metabolic shift. Both in muscle and liver, PHD1 deficiency reduced mitochondrial oxygen consumption through upregulation of PDK1 and PDK4 (Aragones et al 2008, Schneider et al 2009). As a consequence, mitochondrial ROS generation declined, attenuating oxidative damage of the mitochondria (Aragones et al 2008, Schneider et al 2009). By preventing mitochondrial damage, $PHD1^{-/-}$ muscle fibers were able to preserve a certain level of mitochondrial activity despite ischemia, whereas in wild type muscle fibers, extensive mitochondrial damage impeded respiratory activity, resulting in cell death (Figure 6).

Even though no transcriptional changes were detected in glycolytic genes, $PHD1^{-/-}$ muscle fibers showed an enhanced glycolytic flux, likely as energy compensation (Figure 6) (Aragones et al 2008). Of importance, this metabolic switch from oxidative to glycolytic metabolism was observed in normoxic conditions as well, indicating that PHD1 deficiency acts via a preconditioning mechanism in the muscle and the liver: PHD1 deficiency prepares the tissue for a coming ischemic event by increasing its resilience against ischemia. The downside of this metabolic rewiring is that exercise endurance in these $PHD1^{-/-}$ mice was impaired as this relies on oxidative muscle metabolism (Aragones et al 2008).

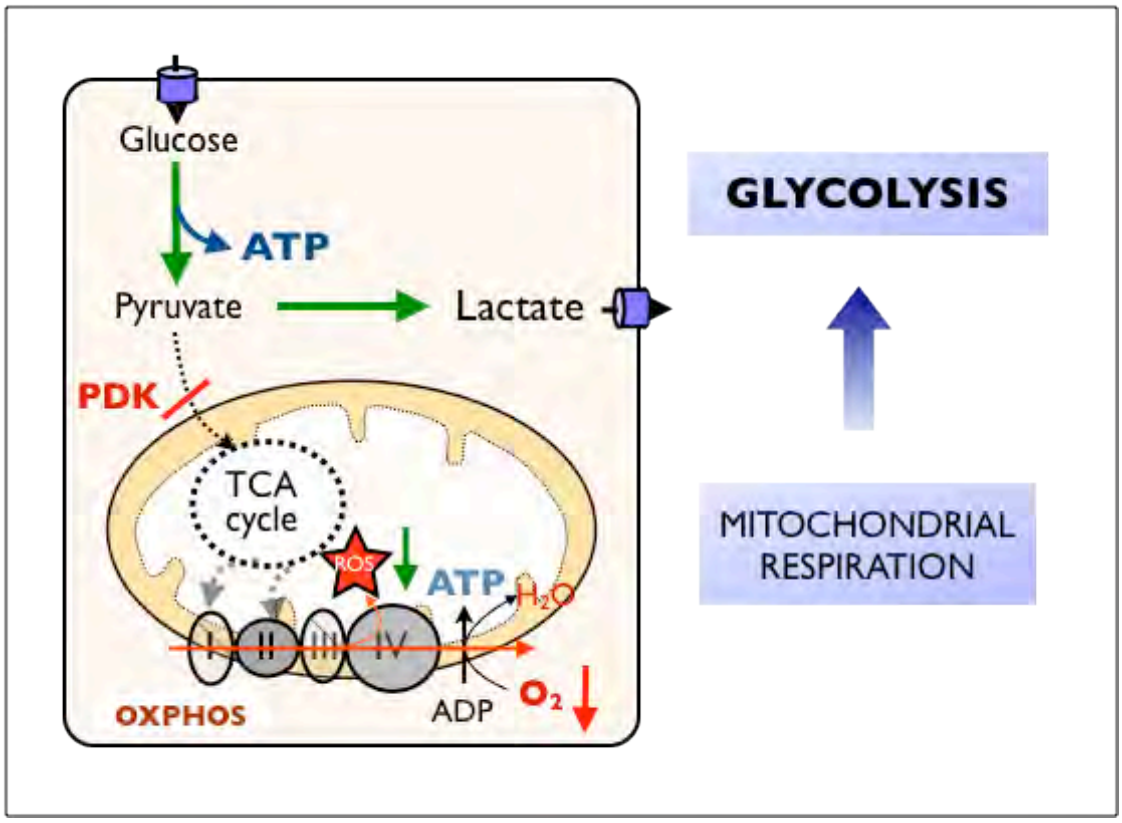

Figure 6: **Metabolic rewiring in PHD1 deficient muscle fibers**

Due to upregulation of PDK, PHD1 deficient muscle fibers reduce the entry of pyruvate into the mitochondria. As a consequence, oxygen consumption as well as mitochondrial ROS production ceases. Increased glycolytic flux and lactate excretion is likely to compensate for the reduced mitochondrial ATP production. In other words, PHD1 deficient muscle fibers show a shift from mitochondrial respiration towards glycolysis. For reasons of simplicity and clarity, the stoichiometry of the reactions has been omitted.

2) PHD2

The role of PHD2 in cellular metabolism has been hardly described, but as the primary regulator of HIF-1α stability, one would assume that inactivation of PHD2 would induce via HIF-1α the above-described shift to anaerobic energy generation. Indeed, brains with a neuron-specific deletion of PHD2 showed enhanced levels of HIF-1α and glycolytic genes in hypoxic conditions (Kunze et al 2012). Also, somatic mutations in PHD2 have been associated with similar metabolic changes in cancer cells (Calvisi et al 2007, Kato et al 2006). Conversely, endothelial PHD2 deficiency indirectly reduced the HIF-1α-induced metabolic changes in tumor cells showing decreased lactate and PDK levels. The lowered HIF-1α levels were the result of a HIF-2α-dependent vessel normalization and improved tumor oxygenation (Mazzone et al 2009). Furthermore, heterozygous PHD2 deficiency protected against the side-toxicity of chemotherapy by mounting an anti-oxidant response (Leite de Oliveira et al 2012).

3) PHD3

A recent paper acknowledged a new role for PHD3 in metabolism. PHD3 was discovered to hydroxylate the glycolytic gene PKM2 (Luo et al 2011). When hydroxylated, PKM2 will via direct binding to HIF-1α in the nucleus, facilitate its transcriptional activity. As both PKM2 and PHD3 are HIF-1α targets, this modulation acts as a feed forward loop in stimulating glycolysis. The nuclear role of PKM2 as transcription modulator goes beyond HIF-1α binding, and needs further study.

iv. Consequences for ischemic diseases

The growing insights into PHDs' major role in maintaining oxygen and metabolic homeostasis prompted scientists to investigate the implication of PHDs in ischemia tolerance and exploring PHD inhibition as a therapeutic

approach in ischemic diseases. Indeed, several studies over the last decade demonstrated that genetic deletion of one the PHD isoforms or pharmacological inhibition of all PHDs protects against ischemia in different organs. A selected list of studies reporting on hypoxic tolerant organs in mice with a genetic deletion of a specific PHD isoform is presented in table 1.

Table 1: Role of PHDs in ischemic tolerance

MOUSE	ORGAN	MECHANISM	REFERENCE
PHD1$^{-/-}$	muscle	Reducing O_2 consumption HIF-2α (>HIF-1α)	(Aragones et al 2008)
	liver	Reducing O_2 consumption HIF-2α?	(Schneider et al 2009)
PHD2$^{+/-}$	muscle	Increased collateral growth NF-κB	(Takeda et al 2011)
PHD2 hypomorph	heart	HIF?	(Hyvarinen et al 2010)
siRNA PHD2	heart	Enhanced purinergic signaling HIF-1α	(Eckle et al 2008)
cardiomyocyte specific PHD2$^{-/-}$	heart	HIF-1α?	(Holscher et al 2011)

Table overviewing a selection of reports, showing that PHD deficiency promotes ischemia tolerance. The genetic mouse model and the organ or tissue in which ischemia was induced is listed, as well as the mechanism of protection and its (putative) molecular mediator.

2.4 Complexity and diversity of the PHD-induced hypoxic response

Given the broad range of oxygen concentrations between physiological oxygen levels and anoxia, the PHD-HIF cascade should allow a graded response. Also, as integrators of cellular homeostasis, PHDs should enable distinct responses adjusted to a variety of stimuli. To provide the necessary fine-tuning of the hypoxia response and shape a cellular outcome suited for the varying contexts of nature, severity and duration of the stimulus, the PHD-HIF pathway exhibits a high degree of flexibility and complexity (Lendahl et al 2009). This diversity is tailored at several levels:

2.4.1 PHD1, 2 AND 3: THREE NON-REDUNDANT PHD ISOFORMS

Worms and flies possess only one PHD isoform (referred to as Egln). This PHD isoform has in vertebrates, with a more advanced cardiorespiratory system, diverged into three different PHD isoforms (Taylor and McElwain 2010). These three PHD isoforms have a homologous C-terminal catalytic domain, but show substantial differences in the N-terminal part. This adds a level of diversity to the hypoxic response in vertebrates as these three isoforms differ in tissue distribution, expression regulation, as well as their preferences for the different HIF-α isoforms. Indeed, genetic mouse studies have demonstrated that the three PHD isoforms are non-redundant and have diverging organ-specific functions. Table 2 shows a summary of some of the observed phenotypes in the different PHD knock-out mice.

Factor inhibiting HIF (FIH) is another member of the same family of 2-oxoglutarate dependent iron(ii)-dioxygenases. FIH hydroxylates in an oxygen-dependent manner a specific aspargine residue on HIF-α that will impede the recruitment of ancillary transcriptional co-activators. This implicates that when FIH loses its activity in hypoxic conditions, HIF-α transcriptional activity will be facilitated (Quaegebeur and Carmeliet 2010). Since FIH exhibits a higher affinity for oxygen than PHDs, FIH will remain active at reduced oxygen

concentrations when PHDs have already lost their activity. In this way, FIH enables an additional fine-tuning of the hypoxic response (Schneider et al 2009).

Table 2 Different phenotypes in genetic knock-out mice of PHD1, 2 and 3.

GENOTYPE	PHENOTYPE
PHD1$^{-/-}$	Protection against limb ischemia, yet increased muscle fatigue due to metabolic shift from mitochondrial respiration towards glycolysis in muscle (Aragones et al 2008)
	Protection against ischemia-reperfusion injury in liver (Schneider et al 2009)
	Reduced mammary gland proliferation due to decreased cyclin D1 levels (Zhang et al 2009)
	Hyperplasia of pulmonary neuroepithelial bodies (Pan et al 2012)
	Protection against colitis due to reduced epithelial apoptosis and enhanced barrier function (Tambuwala et al 2010)
PHD2$^{+/-}$	Normal vascular development; tumor vessel normalization, leading to reduced metastasis and tumor invasiveness due to improved tumor oxygenation (Mazzone et al 2009)
	Reduced cytotoxicity by chemotherapy in healthy tissue (Leite de Oliveira et al 2012)
	Enhanced collateral network in limbs, governing protection against limb ischemia (Takeda et al 2011)
PHD2$^{-/-}$	Congestive heart failure due to increased angiogenesis and

(postnatal)	polycythemia (Minamishima et al 2008, Takeda et al 2007)
PHD2$^{-/-}$ (prenatal)	Embryonic lethality due to placental and heart defects (Takeda et al 2006)
Cardiomyocyte PHD2 deletion	Protection against acute myocardial ischemic injury (Holscher et al 2011)
Adipocyte PHD2 deletion	Protection against diet-induced obesity and improved glucose tolerance (Matsuura et al 2013)
Epithelial PHD2 deletion	Accelerated wound healing due to enhanced migration and proliferation of epithelium (Kalucka et al 2013)
PHD3$^{-/-}$	Hypofunctional sympathoadrenal system and reduced blood pressure (Bishop et al 2008)
	Enhanced innate immunity response and increased mortality in abdominal sepsis (Kiss et al 2012)
	Reduced neutrophil inflammation in colitis (Walmsley et al 2011)
Hepatic PHD3 deletion	Amelioration of diabetes mellitus due to increased insulin sensitivity (Taniguchi et al 2013)
PHD1$^{-/-}$PHD3$^{-/-}$	Viable and fertile with smaller litters and moderate erythrocytosis (Takeda et al 2007)
PHD1$^{-/-}$PHD2$^{-/-}$ postnatal	Premature lethality due to hepatic steatosis and dilated cardiomyopathy (Minamishima et al 2009)
PHD1$^{-/-}$PHD3$^{-/-}$ Hepatic PHD2 deletion	Reactivation of hepatic EPO production (Minamishima and Kaelin 2010)

2.4.2 HIF-1α AND HIF-2α ARE NON-REDUNDANT TRANSCRIPTION FACTORS

That HIF-1α and HIF-2α are non-redundant isoforms is illustrated by the embryonic lethality of both HIF-1$\alpha^{-/-}$ and HIF-2$\alpha^{-/-}$ mice (Majmundar et al 2010). Whereas HIF-1α is expressed throughout the body, the distribution of HIF-2α and HIF-3α is more restricted to certain cell types in heart, lung and brain. How different HIF-α isoforms can induce distinct transcriptional responses is still subject of intense research. Yet, in general it is accepted that HIF-1α governs the classical hypoxic response whereas HIF-2α activates a more limited and specific set of genes and would rather be involved in the protection against oxidative stress (Majmundar et al 2010). HIF-3α is still very little studied and is assumed to act as a negative regulator of the HIF-response.

2.4.3 HIF-INDEPENDENT TARGETS: HYDROXYLATION AND NON-HYDROXYLATION DEPENDENT

Apart from HIF-α hydroxylation, HIF-independent protein targets of PHDs are increasingly being identified, among which members of the canonical nuclear factor kappa B (NF-κB) activation pathway (Chan et al 2009, Cummins et al 2006, Xue et al 2010), activation transcription factor 4 (ATF4) (Hiwatashi et al 2011, Koditz et al 2007), Forkhead box O3 (Foxo3a) (Zheng et al 2014) and others. Similarly to HIF-α regulation, these molecules will accumulate in hypoxia due to inhibition of their proteosomal degradation. In this way, PHDs do not only instruct the hypoxic adaptation, but convolute with biological responses beyond and intertwined with hypoxia such as inflammation, ER stress, etc. Notably, PHDs not necessarily rely on hydroxylation to affect their target proteins: growing evidence points to non-hydroxylation dependent effects of PHDs (Chan et al 2009, Xue et al 2010, Zhang et al 2009), even though the precise nature of these interactions remains obscure.

2.4.4 INTERACTION WITH OTHER SIGNALING CASCADES

The PHD-HIF pathway has an intimate crosstalk with other major gene regulatory pathways, intersecting with diverse biological processes, such as with nutrient metabolism via regulation of 5' adenosine monophosphate-activated protein kinase (AMPK), with protein synthesis, folding and degradation via effects on mTOR and the unfolded protein response (Wouters and Koritzinsky 2008), with apoptosis and cell survival via p53 (Xenaki et al 2008), and many others. Finally, another level of complexity is added by the posttranslational modifications of HIF, as well as transcriptional regulation by microRNA and epigenetic factors (Lendahl et al 2009).

3 WHY SHOULD WE STUDY THE ROLE OF PHDS IN THE BRAIN? - FOOD FOR THOUGHT

PHDs are key regulators of the hypoxic response and enable, via various mechanisms, cellular survival in conditions of insufficient oxygen availability. Still, the role of specific PHD isoforms and their mechanisms of action in different organs as well as their therapeutic potential warrant further investigation. This doctoral thesis aims to investigate the function and the therapeutic potential of the PHDs in the central nervous system. Do PHDs regulate the delicate oxygen and metabolic homeostasis of the brain? Would PHD inhibition be beneficial in conditions where this homeostasis is acutely or chronically disturbed? If so, via which mechanisms would PHD inhibition affect neuronal survival? And finally, is PHD inhibition a valid therapeutic target in neurological disease?

Critical yet delicate oxygen and metabolic homeostasis in the BRAIN

So far, our insights into the biological functions of PHDs in the brain have been rather scarce. Still, one can assume an important role for PHDs, as the brain is putatively the most sensitive organ to any metabolic disturbance. Indeed, in conditions of impaired oxygen or glucose supply such as in hypoglycemia or upon a drop in blood pressure, the brain will be the organ that suffers first and foremost. The brain's tremendous metabolic requirements along with its rather limited reserve capacity is likely to explain this critical dependence on a continuous oxygen and glucose supply.

3.1 The brain is an expensive organ: high oxygen and glucose consumption

Even though the human brain makes up for 2% of the total body weight, it uses up to 20% of the total oxygen consumption in a resting body. This is mainly due to the extraordinary energy demands of normally functioning

neurons. The energetic cost of neurotransmission is high: electrical activity (which comprises restoration of the membrane potential following depolarization, ion pumping to maintain resting potential, synaptic transmitter and vesicle recycling) consumes up to 75% of the cerebral oxygen (Attwell and Laughlin 2001, Harris et al 2012) and will be impaired upon any interruption of oxygen supply (Astrup et al 1981, Hofmeijer and van Putten 2012). Counterintuitively, with an oxygen pressure of only 1-5%, the brain has rather low oxygen levels compared to other systemic organs (Silver and Erecinska 1998).

The high energetic cost of the brain is also reflected in its glucose consumption. The brain, even at rest, is responsible for 20% of total glucose consumption in the human body. In physiological conditions, glucose is the sole energy substrate for the brain (Mergenthaler et al 2013).

3.2 Few defense mechanisms against ischemia

Upon limited blood supply the brain will trigger mechanisms to safeguard cerebral perfusion. However, these act only in a narrow range. At the level of cellular metabolism, neurons have hardly any reserve capacity.

3.2.1 Vascular responses to safeguard cerebral perfusion

Using 20% of the cardiac output, the brain needs to maintain its perfusion within narrow ranges. One of the mechanisms that comes to play when blood pressure drops is cerebrovascular autoregulation: the brain will keep its perfusion constant within certain ranges of systemic blood pressure by adapting the vascular tone of pial and intraparenchymal arteries and arterioles (Aaslid et al 1989). Nevertheless, this protective mechanisms is exhausted rapidly and importantly, compromised in diseases such as diabetes mellitus, hypertension and after ischemic stroke (Jackman and Iadecola 2014).

In order to dynamically match blood supply to energy need, the brain possesses different neurovascular signaling mechanisms. Synaptic activity will evoke an increase in blood flow (functional hyperemia), via the release of vasoactive agents (Attwell et al 2010, Jackman and Iadecola 2014). At the capillary level, an intimate structural and functional interaction between endothelial cells, pericytes, glia and neurons – coined the blood-brain barrier (BBB) – allows for the regulation of microvascular flow, as well as to control the metabolic environment of the parenchym by limiting free molecular transport across this barrier, due to a tightly sealed endothelial layer (Iadecola 2004, Iadecola and Nedergaard 2007, Zlokovic 2008).

These mechanisms of neurovascular coupling are mechanisms that ensure adequate perfusion in physiological conditions, but are often compromised in common pathological conditions such as arterial hypertension and diabetes. This will pave the path for metabolic deregulation, which might ultimately progress to metabolic collapse and neuronal death (Iadecola 2004, Iadecola 2013, Jackman and Iadecola 2014)

3.2.2 Little metabolic reserve capacity

The reason why neurons are believed to be so vulnerable to ischemia is that they rely exclusively on oxidative metabolism for their energy production and are strictly glucose-dependent, yet are believed to have no internal energy stores such as glycogen (Mergenthaler et al 2013). In other words, they have to operate within a small margin of metabolic safety.

Besides disrupting the energy balance, ischemia will also threaten the cellular redox status. Even though this highly oxidative cell produces large amounts of oxygen radicals during oxidative phosphorylation, neurons have proportionally little anti-oxidant defense mechanisms (Bolanos and Almeida 2010). The high levels of redox-active metals (e.g. Fe^{2+}) and polyunsaturated fatty acids together with the fact that neurons are postmitotic cells, which

accumulate DNA damage over time, are additional reasons for neurons to be so particularly prone to oxidative damage (Wang and Michaelis 2010).

All of this is to be found in classical textbooks on brain metabolism. Meanwhile, a lot of these dogmas on bioenergetics have been rephrased or complicated with controversies. Given the extensive link between PHDs and metabolism, in the next paragraph, I will elaborate further on our current understanding of different metabolic pathways in neurons and their surrounding glial cells.

4 BRAIN METABOLISM: A COMPLEX PUZZLE WITH MISSING AND CONTROVERSIAL PIECES

Notwithstanding the growing interest in brain metabolism, it remains fair to say that our current understanding of the role of distinct metabolic pathways in specific physiological (e.g. neuronal activation) and pathophysiological (e.g. ischemia) conditions is rather limited and at times even highly controversial. Whereas in the cancer field metabolic alterations have been extensively integrated with cellular signaling cascades during the last decade, these insights are only in its infancy in the field of brain metabolism.

4.1 Bioenergetics in the brain

“Glucose is the obligate energetic fuel for brain function” (Siesjo 1987). This longstanding dogma perfectly pictures the current controversies in the field. As a first clarification, this dogma implies blood-borne glucose. Neurons and their surrounding glial cells are believed to not only oxidize glucose, but also other mitochondrial substrates, such as glutamine/glutamate or lactate. However, all of these substrates are glucose-derived since the BBB is believed to only allow glucose to cross. Even this statement needs further refinement, as recently the contribution of lactate and ketone bodies in the blood to energy generation in the brain has been acknowledged, albeit only to a limited extent and in certain circumstances (i.e. physical exercise and prolonged fasting respectively) (Lutas and Yellen 2013, van Hall et al 2009).

Another common knowledge in the field of brain metabolism is that neurons rely on oxidative metabolism for energy production. Indeed, being the cells with the highest energy requirements (Howarth et al 2012), they need the most efficient means of ATP production. Remarkably, neurons seem to be unable to upregulate their glycolytic flux to a similar extent as other cells would do in conditions of limited oxygen availability (Herrero-Mendez et al 2009). Not surprisingly then that, next to cardiomyocytes, neurons are the cells with the highest oxygen consumption rate (De Bock et al 2013). This

oxygen consumption is tightly coupled to neural activity, corroborating the belief that synaptic activity is primarily fed by oxidative metabolism in neuronal mitochondria (Hall et al 2012, Harris et al 2012, Lin et al 2010, Rangaraju et al 2014). Nevertheless, glycolysis-derived ATP might be of physiological significance in some processes of neurotransmission such as fast axonal transport of neurotransmitter vesicles (Zala et al 2013), vesicular uptake of glutamate (Ikemoto et al 2003), and glutamate re-uptake (Schousboe et al 2010). Aerobic glycolysis was also postulated to fuel the biomolecular demand of neurons, even though this function is believed to become less important after postnatal brain development (Bauernfeind et al 2014). Simplified and generally speaking, neurons need blood-born glucose and oxygen for energy production

There is often a misconception about the substrates that can be utilized in neurons. The aforementioned dogma is commonly misinterpreted as if glucose would be the only substrate that can fuel mitochondria in neurons. However, it has become clear that neurons oxidize a substantial amount of other substrates besides glucose. Next to glucose, lactate and to a lesser extent glutamine are important fuels for neurons and both are believed to originate from astrocytic metabolism (Mergenthaler et al 2013, Zielke et al 2009). This exchange of metabolites between astrocytes and neurons is imbedded in an intricate functional astrocytic-neuronal relationship in which astrocytes metabolically support their neighboring neurons in various ways (Belanger et al 2011). Not only energy substrates, but also neurotransmitters and glutathione precursors are being passed to neurons (Castro et al 2009, Dringen 2000). Being the cells that line the vessel wall, astrocytes take up glucose from the bloodstream and can store glucose as glycogen in order to build-up an energy back up (Brown and Ransom 2007, Brown and Ransom 2014). As importantly, they will clear the synaptic cleft from accumulating glutamate after neuronal firing via glutamate re-uptake transporters (excitatory amino-acid transporters EAAT1 and EAAT2) (Bak et al 2006b). Two intercellular neuroglial metabolite exchange shuttles have been described

over the last decades: the lactate shuttle and the glutamate-glutamine cycle (Belanger et al 2011). However, ever since, the exact energy source for brain activation (glucose *versus* lactate) has been heavily debated, without any apparent consensus (Belanger et al 2011, Jolivet et al 2010, Mangia et al 2011).

On one side of the debate, there are the believers of the astrocytic-neuronal lactate shuttle (ANLS). This metabolic theory was originally proposed two decades ago by Pellerin and Magistretti (Pellerin and Magistretti 1994) (figure 7). Their data suggested that lactate is the primary energy fuel for neuronal activity. According to their theory, glutamate released in the synaptic cleft following neuronal activation will be taken up by astrocytes and be transformed into glutamine. Since glutamate uptake as well as glutamine synthesis are ATP-dependent processes, glycolysis will rise in astrocytes. The resulting lactate will be released and be taken up by neurons to serve as an oxidative energy substrate (Belanger et al 2011). The astrocytes will also shuttle glutamine to the neurons, where it will be converted into glutamate (the glutamine-glutamate shuttle) (McKenna 2007). This does not only replenish their neurotransmitter pool, but also offers them an additional energy source for oxidation.

Other groups strictly refute this compartmentalized view on brain metabolism based on different arguments. They claim that the predominant cellular fate of glucose during neuronal activation is neurons themselves (Mergenthaler et al 2013). In their views, blood-borne glucose is directly taken up by neurons via their high affinity GLUT-3 transporters (Simpson et al 2007), after which it is predominantly oxidized in the mitochondria (Bak et al 2006a, Hall et al 2012, Hertz et al 2007, Mangia et al 2011).

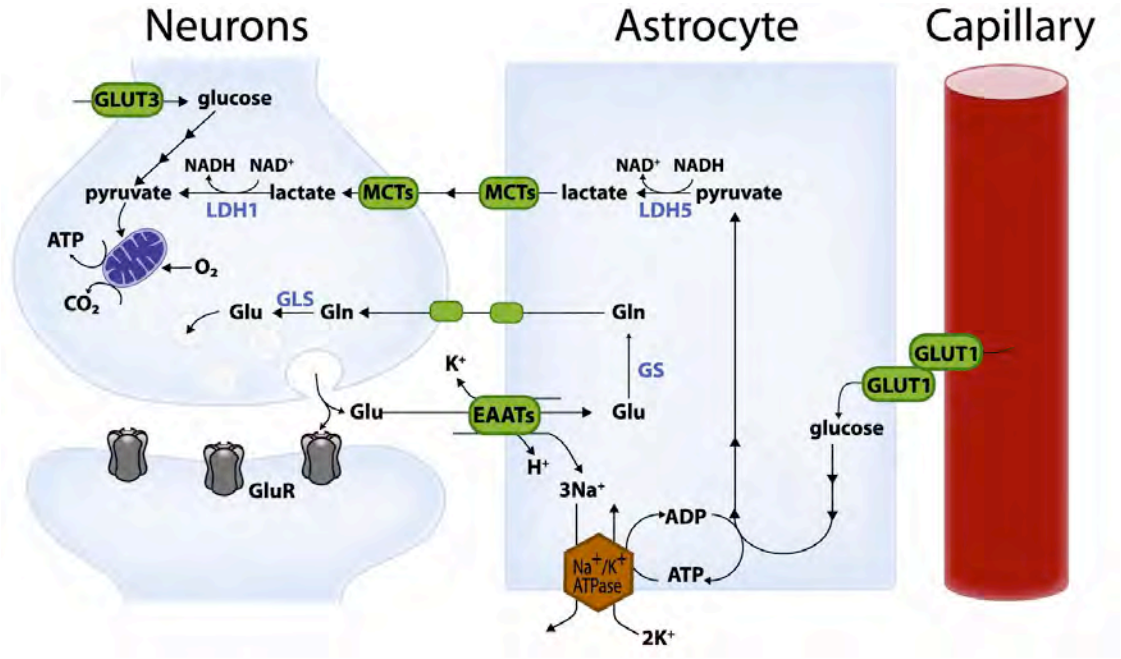

Figure 7: Astrocyte-neuron lactate shuttle

Overview of metabolic pathways involved in the astrocyte-neuron lactate shuttle. Glucose is taken up from the blood in astrocytes via GLUT1 and in neurons via GLUT3. Glutamate (Glu) will after its release as neurotransmitter, be cleared from the synaptic cleft by the astrocytes via glutamate-uptake transporters (excitatory amino acid transporters, EAAT). Since this process is ATP-dependent, astrocytes will increase their glycolytic flux. According to the astrocyte-neuron lactate shuttle hypothesis, the resulting lactate molecules will be taken up by neurons via monocarboxylate transporters (MCT). Via conversion to pyruvate by lactate dehydrogenase (LDH), they can use lactate as an oxidative substrate in their mitochondria. The glutamate taken up in the astrocytes is believed to be recycled via conversion by glutamine synthetase (GS) into glutamine (Gln). Neurons will take up this glutamine and convert it by glutaminases (GLS) to glutamate, which can then be used again as a neurotransmitter (from Bélanger et al., Cell Metab 2011). For reasons of simplicity and clarity, the stoichiometry of the reactions has been omitted.

These two conflicting perspectives also disagree on the fate of glutamate in astrocytes after re-uptake. Since stoichiometrically, the glutamate/glutamine cycle did not appear to occur in a one to one relationship (in which one molecule of glutamate is transformed into one molecule of glutamine in astrocytes), it is commonly believed that in astrocytes glutamate undergoes partial oxidative degradation as well as *de novo* synthesis (Bauer

et al 2012, Dienel 2013, Hertz et al 2007, McKenna 2007). Another level of controversy was added by two recent reports identifying oligodendrocytes (and not astrocytes) as lactate providers for neuronal axons (Funfschilling et al 2012, Lee et al 2012).

The supporters of the astrocytic-neuron lactate shuttle however, state that due to low expression of the aspartate-glutamate carrier in astrocytes, glutamate cannot enter the mitochondria and therefore cannot be oxidized (Berkich et al 2005, McKenna et al 2006, Pardo et al 2011). The resulting low malate-aspartate shuttle activity also prevents the mitochondrial oxidation of reducing equivalents NADH generated during glycolysis, making conversion to lactate necessary in order to replenish NAD^+ (Belanger et al 2011).

In conclusion, after several decades of research, the field of brain metabolism is still clouded by controversies and a general poor understanding on the contribution and regulation of different metabolic pathways. Different experimental and physiological circumstances are likely to explain some of the conflicting results. Combined approaches integrating ^{13}C-labeled metabolic flux measurements with genetic mouse studies of metabolic genes will potentially shed more light on the many outstanding questions.

4.2 Redox homeostasis in neurons

4.2.1 Mitochondria as main site of ROS generation in neurons

The mitochondria are not only the bioenergetic engines of neurons; they also represent a major site of ROS generation. Electrons from reduced substrates are transferred from one redox-complex to another, building up a proton gradient across the mitochondrial membrane. At complex IV (cytochrome c oxidase), oxygen will serve as the final electron acceptor resulting in its reduction to water. Electrons can however escape prematurely, which commonly occurs at complex I and III (Andreyev et al 2005, Lambert and Brand 2004, Turrens 2003). An available oxygen molecule will trap this free electron, leading to the formation of a superoxide radical. More recently, 2-

oxoglutarate dehydrogenase was also acknowledged as an important site of mitochondrial superoxide production (Starkov et al 2004, Tretter and Adam-Vizi 2004).

Under physiological conditions, superoxide radicals are continuously generated in low amounts as normal byproducts of oxidative phosphorylation (Barja 1999, St-Pierre et al 2002). There are however many factors that can influence the rate of superoxide production along the electron transport chain. From a simplistic point of view, one could reasonably guess that oxygen concentration is a determining factor. In extreme conditions this is indeed the case: in the absence of oxygen molecules, no superoxides will be formed. Still, oxygen availability will only be limiting when oxygen tensions drop below 5 μM, which corresponds to almost anoxic conditions (Hoffman et al 2007). In addition, the redox state of the sites of superoxide generation and the mitochondrial membrane potential will also determine the likelihood of premature electron escape (Starkov and Fiskum 2003). In conditions of reduced ATP production and a low NAD^+/NADH ratio, electrons will stall along the redox complexes and easily react with surrounding oxygen molecules. This explains why it is generally believed that hypoxia is associated with an increase in mitochondrial ROS formation (Bolanos et al 2009, Chandel and Schumacker 2000).

Superoxides will immediately be neutralized by the abundant superoxide dismutase 2 (SOD2) enzyme in the mitochondria (Lin and Beal 2006). The resulting hydrogen peroxide (H_2O_2) molecules need further detoxification as they can lead to the generation of highly reactive hydroxyl radicals and peroxynitrite, by metal-catalyzed reduction and interaction with nitric oxide (NO) respectively. When excessively present, these will oxidatively damage different biomolecules: oxidation and inactivation of redox-sensitive mitochondrial proteins (e.g. aconitase, complex I) will interfere with the mitochondrial bioenergetic function. Cumulatively, oxidative mitochondrial DNA strand breaks will disrupt transcription of mitochondrial proteins. Finally, peroxidation of lipids in the mitochondrial membrane will undermine its

structural integrity (Lin and Beal 2006, Starkov 2008). Thus, the mitochondria are not only the source of ROS but are at the same time also heavily under attack by it. This also illustrates how redox and bioenergetic dynamics are in an interactive fashion tied to the functional integrity of the mitochondria.

Besides the mitochondria, other enzymes such as NADPH oxidase, xanthine oxidase and the cyclo-oxygenase pathway can result in ROS generation. However, the extent of their contribution to neuronal ROS generation is not yet fully understood and appears to be limited to specific pathological circumstances (Abramov et al 2007, Bolanos et al 2009).

4.2.2 ROS SCAVENGING CAPACITY IN NEURONS

As described above, there is a continuous production of ROS that need to be detoxified. Oxidative stress and the resulting damage arise when the antioxidant capacity can no longer meet the rate of ROS generation. Although literature on oxidative stress in brain disease is abundant, much less attention has been given to the regulation of antioxidant defense mechanisms.

The role of superoxide dismutase (SOD) in transforming superoxide radicals to H_2O_2 was already mentioned. The two main enzymatic H_2O_2 detoxification systems in neurons are catalase and glutathione peroxidase (GPx). Catalase will convert H_2O_2 to H_2O and O_2, whereas GPx will reduce H_2O_2 to H_2O by using an electron from the co-factor glutathione. Monomeric glutathione (GSH) is a tripeptide (γ-glutamyl-cysteinyl-glycine) that after its oxidation by GPx to glutathione disulfide (GSSG) will have to be recycled by glutathione reductase. Glutathione reductase uses NADPH as reducing equivalent to reduce GSSG to GSH (Figure 8) (Dringen et al 2005). A large body of literature underscores the contribution of these enzymatic anti-oxidant components to the neuronal resilience against oxidative stress *in vitro* and *in vivo* (Aoyama et al 2006, Buckman et al 1993, Esposito et al 2000, Gonzalez-Zulueta et al 1998, Hoehn et al 2003, Jain et al 1991, Junjing et al 2010, Kim et al 2002).

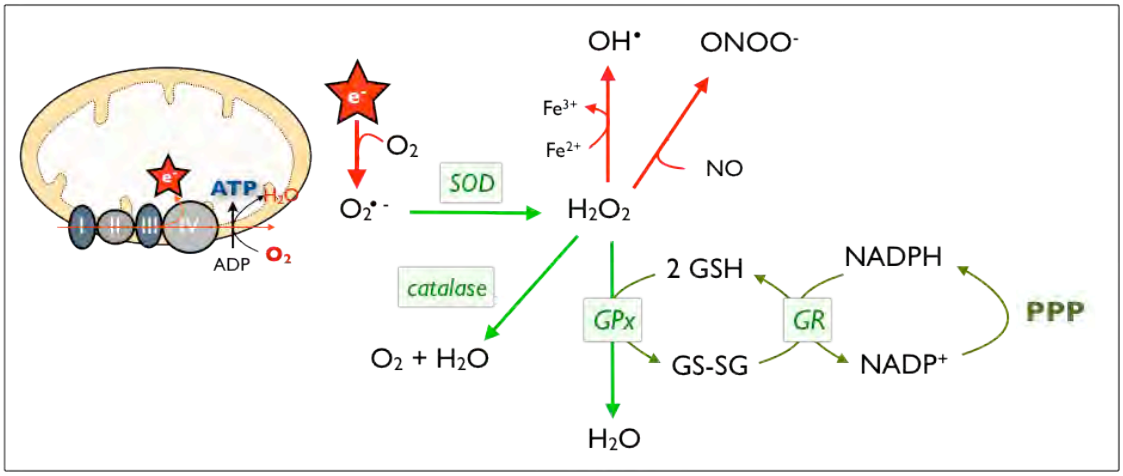

Figure 8: Pathways of ROS scavenging

Electrons that prematurely escape from the electron transport chain react with oxygen molecules resulting in the generation of superoxide radicals ($O_2^{\cdot-}$). Superoxide dismutase (SOD) will rapidly convert this superoxide to hydrogen peroxide (H_2O_2). Two main enzymatic H_2O_2-detoxification systems are present in neurons: catalase transforms H_2O_2 to H_2O and O_2. Glutathione peroxidase (GPx) uses glutathione (GSH) as cofactor to reduce H_2O_2 to H_2O. This will result in the oxidation of GSH to glutathione sulfide (GSSG). To regenerate GSH, glutathione reductase (GR) will use NADPH (generated in the pentose phosphate pathway (PPP)) as reducing equivalent. Excessive amounts of H_2O_2 can exceed the scavenging capacity of these enzymes, giving rise to hydroxyl radicals ($OH^{\cdot}$) and peroxynitrite ($ONOO^-$) that will damage various cellular components due to their highly reactive nature.

The glutathione redox cycling implies that NADPH has to be regenerated. The pentose-phosphate pathway (PPP) is believed to be the main source of NADPH in neurons (Bolanos and Almeida 2010). This metabolic pathway is an alternative route for glucose-6-phosphate besides glycolysis and can be divided into two branches (Figure 9). In the oxidative branch, the oxidation of glucose will lead to the generation of NADPH and of ribose-5-phosphate. The latter can be used for nucleotide synthesis or can be recycled into glycolytic intermediates via the non-oxidative branch of the PPP. Notably, this non-oxidative branch of the PPP is bidirectional and cells that

have high demands for nucleotide precursors will upregulate this branch in the opposite direction to increase the production of ribose sugars (Wamelink et al 2008). A previous report however suggested that the flux through the non-oxidative branch of the PPP is low in neurons (Herrero-Mendez et al 2009). The NADPH generated in the oxPPP can serve different purposes: for antioxidant defense, for lipid synthesis and as a substrate for NADPH oxidase (Stanton 2012, Wamelink et al 2008). In neurons, NADPH is believed to mainly participate in the recycling of glutathione since lipid synthesis drops after the developmental stage of the brain and the expression of NADPH oxidase in neurons is low. Several reports have identified NADPH oxidase as an important source of superoxides in different pathological conditions (Abramov et al 2007, Brennan et al 2009, Cooney et al 2013, Kahles and Brandes 2013), even though it is not understood how the fate of NADPH (glutathione regeneration *versus* NADPH oxidase substrate) is regulated in these circumstances.

The PPP turned out to be of vital importance for neuronal anti-oxidant defense as inhibition of the PPP flux resulted in neuronal apoptosis due to depleted pools of reduced glutathione (Herrero-Mendez et al 2009). This study additionally identified a mechanism via which neurons can ensure an adequate flux through the PPP. Phosphofructokinase-2,6-fructosebiphosphatase (PFKFB3) generates fructose-2,6-biphosphate, which acts as an allosteric activator of phosphofructokinase 1 (PFK-1), a rate-limiting enzyme of glycolysis (Figure 9). Thus, PFKFB3 stimulates PFK-1 activity and hence, directs glucose-6-phosphate into the glycolysis at the expense of the PPP (Eelen et al 2013). In neurons, PFKFB3 protein is nearly undetectable because of its continuous posttranslational degradation due to ubiquitination by the E3 ubiquitine ligase anaphase-promoting complex/cyclosome (APC/C-Cdh1) complex (Herrero-Mendez et al 2009). So, by keeping PFKFB3 low, neurons can ensure adequate NADPH regeneration through the oxPPP. Even though in baseline conditions, the contribution of the oxPPP flux to total glucose oxidation is believed to be rather small, the

oxPPP flux can be upregulated in response to oxidative stress (Ben-Yoseph et al 1996).

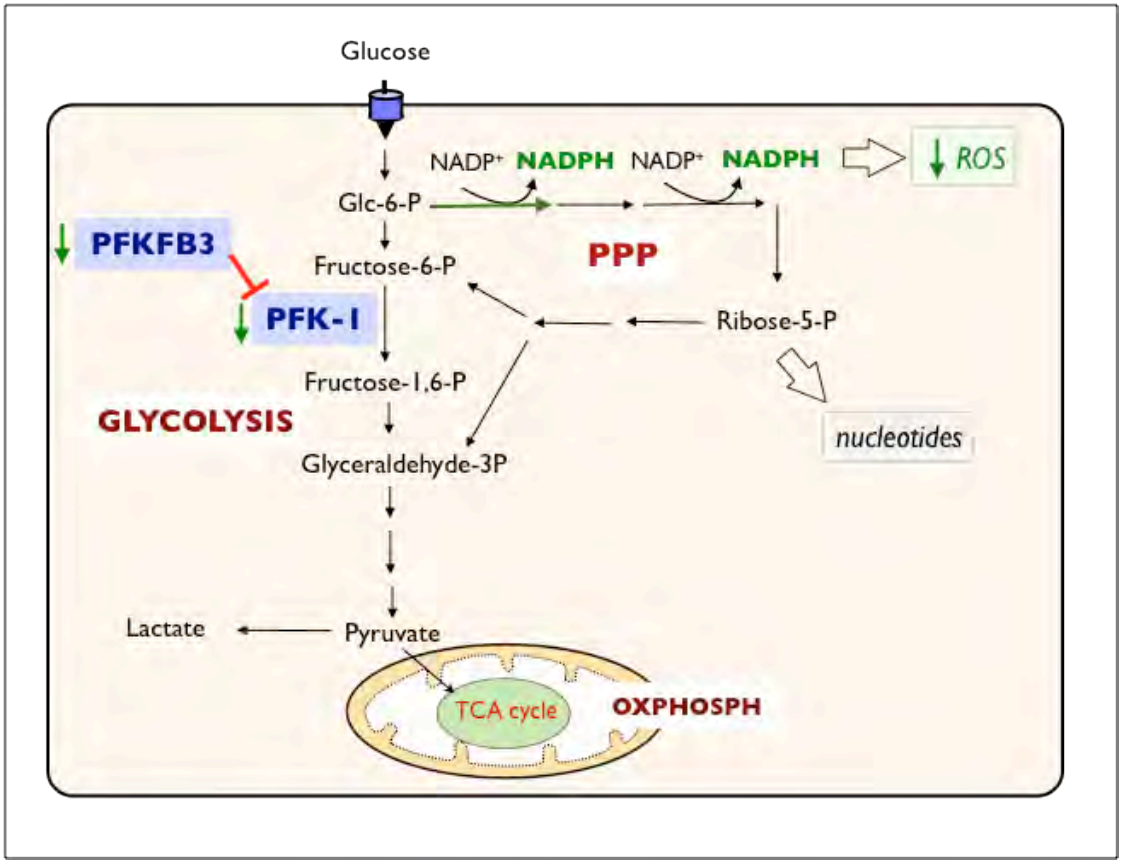

Figure 9: Pentose-phosphate pathway

Overview of glucose metabolism where glucose-6-phosphate (Glc-6-P) can follow two directions: either it undergoes oxidation in the oxidative branch of the PPP. This will lead to the production of nucleotide precursor ribose-5-phosphate (ribose-5-P) and to NADPH, serving as reducing equivalents in NADPH consuming processes such as glutathione reduction by glutathione reductase. In the non-oxidative branch, ribose-5-phopshate can be reconverted to glycolytic intermediates. The activity of phosphofructokinase 1 (PFK1), catalyzing the conversion of fructose-6-phosphate (fructose-6-P) to fructose-1,6-bisphosphate (fructose-1,6-P) will determine the fate of glucose-6-phosphate. Reduced PFK-1 activity will result in glucose-6-phosphate accumulation, facilitating its usage by the PPP. In neurons, low levels of PFKFB3 will lead to low levels of fructose-2,6-bisphosphate and thus low PFK1-activity, resulting in glucose-6-phosphate shifting towards the oxPPP. For reasons of simplicity and clarity, the stoichiometry of the reactions has been omitted.

Other NADPH regenerating enzymes have been described, but have been hardly studied in neurons. Mitochondrial enzymes known for their NADPH regenerating capacity are malic enzyme (ME), isocitrate dehydrogenase 2 (IDH2) and nicotamide nucleotide transhydrogenase (NNT) (Rydstrom 2006). Recently, methylenetetrahydrofolate dehydrogenase, an enzyme involved in folate metabolism was identified as a novel NADPH producer (Fan et al 2014). Since isolated neuronal mitochondria were shown to scavenge H_2O_2 (Zoccarato et al 2004), these enzymes are potential candidates to play a role in mitochondrial NADPH regeneration and mitochondrial redox homeostasis (Arkblad et al 2002, Minich et al 2003, Vogel et al 1998).

5 STROKE & NEURODEGENERATION: TWO BRAIN DISEASES EXEMPLIFYING DEREGULATED OXYGEN HOMEOSTASIS

Acute ischemic stroke and neurodegenerative diseases are common neurological conditions. They are both responsible for a huge burden to our society. Ischemic stroke is the most obvious example of immediate deregulation of oxygen homeostasis. It has however become clear over the last decade that also neurodegenerative disorders are associated with oxygen stress (Quaegebeur and Carmeliet 2010).

5.1 Ischemic stroke

5.1.1 INTRODUCTION

It is nearly impossible to underestimate the medical, social and economical impact of ischemic stroke. The World Health Organization reports 15 million stroke cases per year worldwide, of which 5 million will result in death and another 5 million in severe disability (Broussalis et al 2012). It is currently the second leading cause of death worldwide and the leading cause of disability. Given the aging population, the disease burden is likely only to increase.

Acute ischemic stroke is caused by the occlusion of an artery resulting in oxygen and nutrient deprivation in a certain brain region. This can have devastating consequences: depending on the affected area, a patient can experience acute hemiparesis, severe speech disturbances, loss of vision etc. The only FDA-approved treatment for ischemic stroke, tissue plasminogen activator (t-PA) aims at recanalization of the occluded vessel, but due to a narrow therapeutic time window this therapy reaches less than 10% of the stroke patients. Therefore, this devastating disease asks for further research into novel therapeutic avenues.

5.1.2 PATHOPHYSIOLOGY

a. Arterial occlusion

A stroke arises when an artery is acutely occluded either by *in situ* thrombus formation or by an embolus originating from the heart, aortic arch or carotid artery. Established treatable risk factors for ischemic stroke are diabetes mellitus, hypercholesterolemia, arterial hypertension, tobacco usage, cardiac disease (atrial fibrillation, ischemic cardiomyopathy, valvular disease) and carotid stenosis (Moskowitz et al 2010). Cardiac disease and carotid stenosis will increase the risk of emboli formation and can be associated with chronic cerebral hypoperfusion, while the other risk factors will compromise cerebral blood flow by inducing both structural and functional vessel wall abnormalities (Moskowitz et al 2010). Atherosclerosis can be found in the wall of larger arteries, whereas smaller intracerebral arterioles can be affected by lipohyalinosis. Risk factors such as diabetes and hypertension will also interfere with the functional regulation of cerebral blood flow at different levels: cerebrovascular autoregulation at the level of large arteries, neurovascular coupling at the level of arterioles and microvascular flow regulation at the level of the blood-brain barrier might become impaired, preventing the brain's ability to respond to changes in blood pressure and neural activity (Iadecola and Davisson 2008).

b. The ischemic cascade in the penumbra

Following the acute arterial occlusion, the processes that lead up to ischemic damage will evolve in a progressive fashion (from minutes to even days). A brain region having its blood supply blocked will not undergo the same blood flow deficit homogeneously. Due to a certain extent of collateral flow, the residual blood flow in the periphery of the ischemic area will be higher than in the core. In the core region of the stroke, residual blood flow will be so low that neurons undergo immediate necrotic death within minutes. When blood flow drops to less than 20% of its normal level, acute ATP depletion will prevent

neurons from maintaining their ion homeostasis and energy balance (Astrup et al 1981, Lo 2008). Anoxic depolarization following ion pump failure will result in cytotoxic edema and rupture of the cell membrane.

Surrounding this core of irreversibly lost tissue, there is an area that only suffers moderate blood flow reduction (20-40% of normal blood flow). Here, neurons become dysfunctional, but remain viable for a certain period of time. The moderate ischemia will preclude neurons from firing action potentials (with electrical silence as a consequence), but will still allow ion pump function to a certain extent. This region was named the penumbra, referring to the astronomical term indicating the area between complete illumination and complete shadow (Astrup et al 1981). This area is of therapeutic interest because it is salvageable: within a certain time window it can be saved by either reperfusion and/or neuroprotective strategies (Moskowitz et al 2010).

However, when the blood flow deficit persists, penumbral cells will succumb to a complex interplay of deleterious processes. As a consequence, the penumbra will shrink in size over time, adding to the necrotic core of stroke. Extensive research from the last three decades has identified the molecular nature of different events that occur in this so-called “ischemic cascade”. Pivotal effectors of neuronal death are glutamate excitotoxicity, peri-infarct depolarization, calcium overload, mitochondrial dysfunction, oxidative stress and programmed cell death (Dirnagl et al 1999, Lo et al 2003, Tymianski 2011). Simplified, due to the lack of oxygen, mitochondrial ATP production drops. This will increase the formation of oxygen radicals in the mitochondria, which will attack the surrounding proteins, lipids and nucleic acids. Due to ROS-inactivation of mitochondrial enzymes, mitochondrial function is further compromised, creating a vicious cycle between ROS generation and mitochondrial bioenergetics (Hertz 2008).

The disturbed energetic balance will also cause a surge in extracellular glutamate levels, since impaired ion pump function will reverse the glutamate transporters (Rossi et al 2000) and will depolarize the membrane resulting in glutamate release. The rising extracellular concentration of glutamate and the

resulting chronic stimulation of the N-methyl-D-aspartate (NMDA) receptor (acting as a cation channel) will cause additional membrane depolarization and overload neurons with calcium. Toxic intracellular calcium concentrations will further aggravate mitochondrial dysfunction and will activate additional sources of ROS and reactive nitrogen species generation such as NADPH oxidase and neuronal NO synthase. All these processes will in an interactive fashion activate effectors of programmed cell death (Dirnagl et al 1999, Lo et al 2003, Moskowitz et al 2010).

5.1.3 THERAPEUTIC OPTIONS

Theoretically, three different approaches exist to treat acute ischemic stroke: restoring the blood flow to the ischemic tissue (reperfusion), increasing the resilience of the surviving penumbral neurons against further ischemic damage (neuroprotection) and facilitating recovery by enhancing repair mechanisms (repair). Given the scope of this literature study, the latter will not be further discussed.

a. Reperfusion

Recanalization is currently the only clinically implemented therapy (Jauch et al 2013). Either by lysing the clot with t-PA (Wardlaw et al 2012), or by mechanically retrieving the thrombus with more experimental endovascular techniques (Ciccone et al 2013, Lansberg et al 2012), this approach aims at removing the thrombus. This will restore the blood flow to the ischemic penumbra, halting further ischemic death. Given the ongoing ischemic cascade in the penumbral tissue, thrombolysis is only beneficial in a narrow therapeutic time window after stroke onset (4.5 hours) and the outcome inversely correlates with the interval between onset and thrombolytic therapy (Khatri et al 2009, Lees et al 2010). Major efforts are being done to identify this penumbra area in stroke patients, as this would select patients that have most chance of benefitting from thrombolytic therapies (Albers et al 2006). Current

clinical neuroimaging tools allow to detect the discrepancy between the area of perfusion deficit and the area of cytotoxic edema, which reflects the ischemic penumbra (Straka et al 2010).

b. Neuroprotection

Neuroprotective therapies refer to any intervention that improves the brain's intrinsic resilience against ischemia (Ginsberg 2008). Similar to the reperfusion strategy, this approach aims at rescuing the penumbral area and therefore, a certain therapeutic time window needs to be considered as well. Extensive research over the past three decades has identified the molecular underpinnings of the ischemic cascade, bringing forward viable targets for neuroprotection in stroke treatment. The central role for ROS, calcium overload, and glutamate excitotoxicity has rationalized preclinical studies with ROS scavengers, calcium channel blockers and glutamate antagonists. All of these drugs showed a beneficial effect on stroke size in rodent models in various studies, further substantiating the contribution of these processes to ischemic damage (Ginsberg 2008, Ginsberg 2009).

In the clinical context, the combination of a neuroprotective drug and thrombolysis might synergistically improve stroke outcome: a neuroprotective drug may prolong the therapeutic window for thrombolytic drugs (by stabilizing the penumbra until reperfusion occurs) and might attenuate the reperfusion injury and antagonize some of the detrimental side effects of tPA (e.g. stimulation of NMDA-receptor signaling and increased blood-brain-barrier permeability) (Ginsberg 2008).

Despite encouraging data from experimental animal models, however, decades of research into neuroprotective strategies have faced serious issues of translational failure (see also chapter V). This illustrates the compelling need for a more integrated and profound understanding of the ischemic cascade and possible strategies to interrogate the stroke process.

5.1.4 STROKE RESEARCH IN ANIMAL MODELS

During the last four decades, rodents have been extensively used to model focal brain ischemia. Each of the different models has its own advantages and limitations. Most often, the middle cerebral artery is occluded via either an intraluminal approach or via external suturing. The latter requires a craniotomy to visualize the tree of the middle cerebral artery, which is then permanently ligated (Robinson et al 1975). Depending on the location of suturing (proximal or distal to the origin of the perforating arteries supplying the striatum), the striatum is also involved in the ischemic injury (Kuraoka et al 2009). While rather invasive given the need for craniotomy, this model allows to generate highly reproducible stroke sizes, which are not influenced by anatomic differences in the circle of Willis (Kanemitsu et al 2002, Majid et al 2000, Schmid-Elsaesser et al 1998).

The intraluminal filament model provides a less invasive way of occluding the middle cerebral artery. A filament is introduced via the internal carotid artery until it reaches and occludes the proximal middle cerebral artery (Longa et al 1989). This model allows the induction of transient cerebral ischemia when retracting the filament. As a drawback, this reperfusion model often results in highly variable stroke sizes, where residual blood flow is likely to be a crucial determinant (Kanemitsu et al 2002, Majid et al 2000, Schmid-Elsaesser et al 1998).

Whereas a neurological examination in rats allows a more detailed testing than in mice, stroke studies in mice facilitated the exploration of the function of various genes in the pathophysiology of ischemic injury by using genetically engineered mice (Canazza et al 2014). In most studies only male animals are used to exclude the estrogen-mediated protective effect as an additional cause of variability (Hurn and Macrae 2000). Also a pure background strain is crucial, as many studies have pointed to a strain-dependent vulnerability to brain ischemia (Barone et al 1993, Connolly et al 1996, Fujii et al 1997, Majid et al 2000).

The many translational failures in the clinical setting have raised skepticism about the relevance of these rodent models for the treatment of acute ischemic stroke. Indeed, acute ischemic stroke in humans is a multifactorial heterogeneous disease occurring in a mostly aged population with different comorbidities such as hypertension and diabetes, each of them also affecting the cerebral vasculature. Mice exposed to middle cerebral artery ligation are on the contrary gender- and strain-matched, young-adult mice, representing a homogeneous population - very much unlike the human population suffering from stroke. Therefore, additional studies in aged mice with diabetes or hypertension will be crucial to extend the relevance of our existing stroke models (Liu and McCullough 2011).

Nevertheless, a body of literature has also indicated that models of brain ischemia in rodents have yielded crucial information for our understanding of the cellular and molecular events of the ischemic cascade as well as for identifying potential therapeutic targets (Ginsberg 2008). One example of an important parallel between the two species is the existence of a penumbra. The hemodynamic definition of the penumbra, where residual blood flow is between 20-40% of the contralateral hemisphere, is similar between rodents and humans (Belayev et al 1997, Heiss et al 1999).

5.2 Neurodegeneration

Next to brain ischemia, where a deregulated oxygen homeostasis is a clear characteristic, also neurodegenerative diseases are associated with an oxygen imbalance, exemplified by the vascular and metabolic alterations observed both in patients as well as in animal models. Evidence for oxidative stress and energy insufficiency is abundant in most neurodegenerative diseases (Quaegebeur and Carmeliet 2010). Vascular abnormalities such as an impaired angiogenic response, neurovascular uncoupling and BBB dysfunction (all likely associated with chronic hypoxia) have extensively been described, especially in Alzheimer's disease and amyotrophic lateral sclerosis (Quaegebeur et al 2011). Whether or not these changes are the cause or the consequence of the disease course, it should be clear that also neurodegenerative diseases involve processes that may be affected by PHD inhibition, pinpointing PHDs also here as interesting therapeutic targets (Quaegebeur and Carmeliet 2010).

6 PHD INHIBITION AS A POTENTIAL NEUROPROTECTIVE TARGET?

6.1 Expression of PHDs in the central nervous system

Prior to any discussion on PHD inhibition as a potential therapeutic target in brain diseases, it is important to pursue the expression pattern of PHDs, as the different isoforms are known to have a tissue-specific distribution. All three PHD isoforms are expressed in the mammalian brain (Lieb et al 2002), but show a rather heterogeneous distribution pattern. PHD1 is, at least at mRNA level, the most abundant isoform with high levels in the hippocampus, cortex and olfactory bulb. PHD2 is also expressed throughout the brain, especially in the cortex and hippocampus. PHD3 expression is remarkably low in the adult brain (Lein et al 2007).

6.2 Lessons from hypoxic preconditioning and ischemic tolerance in the brain

Hypoxia and oxidative stress can, when prolonged and severe, result in neuronal death. However, it has been known for some time that exposing the brain to a controlled stressful stimulus, such as oxygen deprivation, to an extent that neuronal function is slightly impaired, yet not irreversibly damaged, elicits a protective response. In this way, the brain acquires a state of ischemic or hypoxia tolerance and will be protected against a subsequent lethal stimulus (Gidday 2006). This phenomenon is known as ischemic brain preconditioning and has been documented in other organs as well. This concept is of great medical interest as it might prove to be a preventive strategy in high-risk conditions for cerebrovascular events such as a transient ischemic attack or subarachnoid hemorrhage (Dirnagl et al 2009). At first glance, hypoxic preconditioning is not implicated in the field of neurodegenerative disorders given their chronic nature. Nevertheless, an in-depth molecular understanding of preconditioning may allow the identification

of novel neuroprotective players, which could represent attractive disease candidates in both stroke and neurodegeneration (Quaegebeur and Carmeliet 2010).

A preconditioning, non-lethal, stimulus is known to profoundly alter transcriptional output in the brain (Bernaudin et al 2002, Stenzel-Poore et al 2003). The HIF family represents one of the most studied hypoxia-sensitive transcription factors involved in this genetic reprogramming (Correia and Moreira 2009, Harten et al 2009). Both *in vitro* and *in vivo* studies show increased levels of HIF-1α and its downstream targets VEGF, erythropoietin and glycolytic enzymes upon exposure to cellular stress (Baranova et al 2007, Sharp and Bernaudin 2004). Given the well-documented neurotrophic effects of the effector molecules VEGF (Quaegebeur et al 2011) and erythropoietin (Brines and Cerami 2005), HIF-1α is arguably pinpointed as moderator of neuroprotection. Further *in vivo* characterization of the role of HIF-1α in acute cerebral ischemia yields, however, conflicting data (Baranova et al 2007, Chen et al 2009, Helton et al 2005). Pharmacological manipulation of HIF-1α also demonstrates diverging effects on stroke outcome (Baranova et al 2007, Chen et al 2009, Chen et al 2008a, Helton et al 2005, Ratan et al 2008, Siddiq et al 2005, Zhou et al 2008). This may be related to the dual activity of HIF-1α, which not only activates survival-promoting pathways, but also triggers pro-apoptotic proteins such as Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) and p53-upregulated mediator of apoptosis (PUMA) (Chen et al 2008b). Taken together, the overall effect of HIF-1α on neuronal cell fate appears to be context specific and requires further study.

6.3 PHD inhibition: the past, the present and the future

As HIF-1α levels are mainly regulated by the activity of the PHDs, inhibition of PHDs has become an attractive strategy in preconditioning therapy. Current available PHD inhibitors act as iron chelators or 2-oxoglutarate analogs, depleting the enzyme cofactor iron or competing with its cosubstrate 2-

oxoglutarate, respectively (Fraisl et al. 2009). The neuroprotective effects of these agents have been validated in preclinical stroke models (Baranova et al 2007, Freret et al 2006, Hamrick et al 2005, Li et al 2008, Nagel et al 2011, Ogle et al 2012, Reischl et al 2014, Siddiq et al 2005), with in some reports evidence for a, at least partially, HIF-1α-mediated effect (Baranova et al 2007, Hamrick et al 2005, Ogle et al 2012). However, as all these studies have been rather descriptive, the precise mechanism of protection remains obscure. Interestingly, a beneficial effect of post-stroke administration was reported as well (Nagel et al 2011, Ogle et al 2012, Reischl et al 2014).

Inspired by the neuroprotective role of the PHD/HIF pathway in brain ischemia, PHD inhibitors are assessed as treatment design for neurodegenerative disorders as well. Already in 1991, clinical efficacy of iron chelators was tested in Alzheimer's disease patients (Crapper McLachlan et al 1991). Desferrioxamine, another iron chelator, was reported to slow down neurodegeneration in rodent models of Parkinson's disease (Ben-Shachar et al 1991, Lan and Jiang 1997). More recently, novel iron chelating drugs resulted in a survival advantage in an amyotrophic lateral sclerosis (ALS) mouse model (Kupershmidt et al 2009).

Overall, the fact that the PHD-HIF pathway is a putative player in cerebral ischemic preconditioning together with the numerous observations of neuroprotective properties of various aspecific PHD inhibitors, render PHD inhibition an attractive approach in the treatment of diverse neurological conditions. However, an important setback of these drugs is that they lack specificity. Indeed, iron chelating drugs will not only inhibit PHDs, but all other iron-dependent enzymes as well. The resulting iron depletion will also lead to anemia. The inhibition by 2-oxoglutarate analogues is not specific for PHDs either as more than 50 2-oxoglutarate dioxygenases have been described, which will also be inhibited by these drugs (Fraisl et al 2009, Loenarz and Schofield 2008).

Why would this lack of specificity be a concern for therapeutic utility in a clinical setting? Their off-target effects might bring about an unfavorable

safety profile and complicate their clinical use. Ideally, inhibitors of specific PHD iso-enzymes would allow us to refine the concept of PHD inhibition by specifically targeting a disease state without causing unwanted effects. Especially PHD1- and PHD3-specific inhibitors might be well tolerated given the absence of major physiological deficits in $PHD1^{-/-}$ and $PHD3^{-/-}$ mice (Aragones et al 2008, Taniguchi et al 2013). Furthermore, the lack of specificity confounds the role and downstream effects of specific PHD isoforms in neuronal injury. Therefore, *in vivo* analyses of the disease- and cell-specific role of PHD are essential in pursuing the clinical validation of PHD inhibitors. So far, a single *in vitro* study implicated PHD1 in the neuroprotective response to oxidative stress (Siddiq et al 2009), two studies pinpointed PHD2 inhibition as a potential target in transient brain ischemia (Chen et al 2012, Kunze et al 2012) and PHD3 was linked to neuronal apoptosis during development (Bishop et al 2008, Lee et al 2005).

In conclusion, an important role for PHDs in the brain can be assumed, and a growing body of evidence points to the neuroprotective potential of PHD inhibition. However, important gaps in our understanding have to be resolved before this promising preclinical therapeutic target will reach human bedside.

Chapter II

AIMS

Organisms have developed a variety of mechanisms to sense, transduce and regulate adaptive responses to a shortage in oxygen. At the cellular level, the prolyl hydroxylase domain proteins (PHDs) take on a leading role in maintaining this oxygen homeostasis.

Previous work of the host laboratory showed that PHD1 deficient muscle is resistant towards limb ischemia. Interestingly, this ischemia tolerance was attributed to a shift from aerobic metabolism to anaerobic glycolysis. The brain, as the largest consumer of oxygen and nutrients, is a very expensive organ with a delicate metabolic balance. Disturbances in this homeostasis can result in devastating brain diseases such as ischemic stroke and might contribute to neurodegenerative disorders. Yet, surprisingly little is known about the functions of the different PHD isoforms in the brain and even less about whether and how modulation of metabolic pathways could affect neuronal injury.

This prompted a number of outstanding questions on the function of PHDs in brain ischemia: would PHD1 deletion also confer ischemia tolerance in the brain? What would this implicate for diseases such as stroke and neurodegeneration? And even more tantalizing, would a similar metabolic shift as seen in PHD1 deficient muscle also protect against neuronal injury?

We formulated the specific aims for this doctoral work as follows:

- What is the effect of PHD1 deficiency on infarct size in a model of stroke in rodents? Are the potential effects PHD1-specific, or do PHD2 and PHD3 deficiency affect stroke size as well?

- Via which mechanisms and cellular mediators does loss of PHD1 impact on brain ischemia? More specifically, given the metabolic phenotype in the PHD1 deficient muscle, what is the effect of PHD1 deletion on neuronal metabolism? How does this relate to the known effects of PHD1 deficiency on metabolism and does this provide us with additional insights into neuronal metabolism?
- Does the effect of PHD1 deficiency in ischemic stroke have translational potential towards new therapeutic strategies?
- Do the findings have implications for neurodegenerative diseases as well?

Chapter III

MATERIALS & METHODS

1. MOUSE STRAINS USED IN THIS STUDY

PHD1$^{-/-}$ (Aragones et al 2008), PHD2$^{lox/lox}$ (Mazzone et al 2009) and PHD3$^{-/-}$ mice (Bishop et al 2008) were previously generated in the lab. The Nestin-Cre mice were on a C57/Bl6 background and were a kind gift from Dr. R. Klein (MPI, Germany). Nestin-Cre mice were intercrossed with PHD2$^{lox/lox}$ mice. Nestin Cre^{+} PHD2$^{lox/lox}$ mice were compared with Nestin Cre^{-} PHD2$^{lox/lox}$ mice. Mice overexpressing human mutant SOD1^{G93A} were provided by Dr. C. Kunst. They were backcrossed for more than ten generations into a FVB background. The genetic background of the PHD1$^{-/-}$ SOD1^{G93A} mice was FVB(50%)/129S6(25%)/swiss(25%). The genotype of these transgenic animal models was determined by PCR. Wild type C57/Bl6 mice were obtained from the KU Leuven animal facility. For each transgenic mouse line, littermates or age-matched wild type control mice were used. Housing and experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Leuven, Belgium.

2. INDUCTION OF ISCHEMIC STROKE VIA PERMANENT MIDDLE CEREBRAL ARTERY OCCLUSION

Brain ischemia was induced in 8-11 weeks old male wild type and PHD1$^{-/-}$ mice using the permanent middle cerebral artery occlusion (pMCAO) model as previously described (Kuraoka et al 2009). Both C57Bl/6 and 129S6 backgrounds were used, as indicated. Under the surgical microscope, a skin incision was made between the left orbit and tragus. The zygomatic arch was removed and temporal muscle retracted laterally. A 2-mm burr hole was made

with a microdrill through the outer surface of the semitranslucent skull over the visually identified middle cerebral artery. The dura was carefully opened and the M1 branch of the MCA exposed and ligated producing a permanent ligation of the distal middle cerebral artery. The wound was sutured and temperature was controlled by keeping the mice on a heating plate at 37 °C until they regained full consciousness.

3. HISTOLOGICAL ANALYSIS OF INFARCT SIZE

At 24 hours after ischemia, brains were removed, sliced coronally at 1 mm intervals and stained by immersion in vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC) for 30 minutes. TTC will be reduced by active mitochondrial dehydrogenases, indicative of viable tissue, resulting in the formation of a deep-red product (formazan) (Bederson et al 1986). For each coronal slice the unstained portion was delineated, and infarction area was calculated as the ratio of the unstained area (white) over the total bihemispheric area with Image J.

4. ADHESIVE TAPE REMOVAL TEST

The performance in the adhesive removal test was used as a functional parameter to monitor the functional impact of the stroke as previously described (Bouet et al 2009). Mice were trained during 7 days prior to the stroke to avoid any training effect. A 3mm x 4mm piece of tape was attached to both forepaws. Time to sensation and to removal was recorded for both paws. Three separate measurements were done at day 1, 4, 8 and 11 post-stroke.

5. MORPHOLOGICAL AND HISTOLOGICAL ANALYSIS OF BRAIN VASCULATURE

To compare large arteries and arterioles in wild type and PHD1$^{-/-}$ mice, mice were perfused transcardially with saline followed by 4% PFA. Brains were

dissected and post-fixed in 4% PFA overnight at 4°C, cryoprotected in sucrose 20% and embedded in optimal cutting temperature (OCT) embedding medium. Serial coronal cryosections of 10µm thickness were incubated overnight with the primary antibody anti-αSMA-Cy3 (C6198, Sigma, 1/500). Slices were mounted with Prolong Gold + DAPI (Dako).

To assess vessel perfusion, wild type and PHD1$^{-/-}$ mice were injected via the tail vein with fluorescein isothiocyanate (FITC)- conjugated dextran (molecular weight 2x10^6 Da, Sigma) shortly before transcardial perfusion, and were thereafter processed for cryosectioning.

The visualization of the pial collateral circulation in wild type and PHD1$^{-/-}$ mice was done via vascular corrosion casting as previously described (Krucker et al 2006, Zhang et al 2010). The thoracic aorta was cannulated retrogradely and 1 ml of freshly prepared polyurethane resin (PU4ii, VasQtec) was infused into the cerebral vasculature. The PU4ii mixture was freshly prepared by adding 0.8 g of hardener (VasQtec) to PU4ii diluted in dimethylethylketone, immediately before the injection. Following resin injection, mice were perfused with 4% PFA. After 5 days at room temperature to ensure polymerisation of the resin, brains were dissected and were repeatedly rinsed in 30% KOH

Images of the corrosion casting were captured by Zeiss LSM 780 confocal microscope (Carl Zeiss). The number of collaterals was counted manually per hemisphere. Collaterals were defined as vessels connecting the tree of the anterior cerebral artery with the tree of the middle cerebral artery, or connecting one arteriole from the middle cerebral artery with an arteriole from the same vascular tree.

6. ISOLATION AND CULTURE OF MURINE CORTICAL NEURONS

Cultures of cortical neurons were prepared as previously described. Briefly, neuron cultures from WT and PHD1$^{-/-}$ mice were prepared from the cortices of embryonic day 14.5-15.5 mice as described previously (Thathiah et al 2009)

and maintained in serum-free NeuroBasal containing 25mM glucose (Invitrogen), supplemented with 0.5mM glutamine and B27 supplement (Invitrogen, 1/50). After 3 days in culture, 8μM cytosine arabinoside (Sigma) was added to prevent non-neuronal proliferation. These cultures contained >98% $MAP2^+$ neurons after 7 days in culture, as confirmed by immunostaining.

7. LENTIVIRAL TRANSDUCTIONS

Lentiviral shRNA vectors against glucose-6-phosphate dehydrogenase (G6PD), PHD2, PHD3, HIF-1α and HIF-2α, were ordered from the TRC 1.5 lentivirus-based shRNA library (Sigma) consisting of almost 200,000 pre-cloned shRNA vectors targeting more than 22,000 human and 20,000 mouse genes. The shRNA ID nummers were TRCN0000041443 and TRCN0000041447 for G6PD, TRCN0000232222 for HIF-1α, TRCN0000082306 for HIF-2α, TRCN0000009740 for PHD2, TRCN0000009753 for PHD3. A similar nonsense scrambled shRNA sequence was used as a negative control for all these experiments. Production of lentiviruses by transfection into 293T cells was as described (Carlotti et al 2004). After 2 days in culture, the cultured neurons were exposed overnight to a multiplicity of infection of 10 after which the medium was refreshed. Knockdown efficiency was assessed 4 days after transduction by qPCR.

8. OXYGEN-NUTRIENT DEPRIVATION ASSAY IN VITRO

After 7 days in culture, neurons were exposed for 2 hours to 0.1% oxygen in nutrient-deprived medium (Dulbeco's Modified Essential Medium (D5030, Sigma) lacking glucose, glutamine and pyruvate, supplemented with 7.5 mM $NaHCO_3^-$ and 5 mM HEPES and adjusted to pH of 7). After this period of oxygen-nutrient deprivation, neurons were re-exposed to ambient air and regular nutrient-containing culture medium (neurobasal medium supplemented with 0.5 mM glutamine and B27 supplement minus anti-

oxidants, both from Invitrogen). Leaving out anti-oxidants in the medium prevented the masking of a neuron-intrinsic improvement of ROS detoxification, which is crucial in all neuronal cell death assays relating to oxidative stress. Neuronal cell death was quantified 24 hours later by measuring LDH release in the culture medium using the Cytotoxicity Detection Kit (Roche applied sciences). LDH release after maximal lysis of control wells (with Triton-X) was used to calculate the relative cell death.

9. METABOLIC FLUX ASSAYS

Metabolic fluxes were measured using radioactive labeled tracer glucose, glutamine and lactate (all tracers were from American Radiolabeled Chemicals). All fluxes were corrected for the specific activity.

Glycolysis was measured as $^{3}H_2O$ formation using [5-^{3}H]-D-glucose as previously described (De Bock et al 2013). Briefly, at day *in vitro* (DIV) 7, cortical neurons were incubated for 2 hours with medium containing 5 mM glucose and labeled 5-^{3}H-glucose (0.5 μCi/mmol). After this incubation period, the medium was transferred to glass vials, which were closed with rubber stoppers each containing a hanging well with a soaked (200μl Milli-Q water) filter paper (1cm x 6cm) to capture the evaporated $^{3}H_2O$. The vials were incubated for 48 hours at 37°C. Afterwards, the filter papers were transferred to scintillation vials containing 5ml of scintillation fluid (Lumasafe 3087, Perkin Elmer) and counted with the Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer). A correction factor of 22.6 was used to correct the glycolysis flux for the percentage of $^{3}H_2O$ captured by the filtered paper. This correction factor has been determined in earlier pilot experiments in the host laboratory.

Glucose oxidation was assessed as $^{14}CO_2$ formation using [6-^{14}C]-D-glucose as previously described (De Bock et al 2013). At DIV7, cortical neurons were incubated for 2 hours with medium containing labeled [6-^{14}C]-D-glucose (1 μCi/mmol). Perchloric acid ($HClO_4$) (250μl, 3M) was used to stop cellular metabolism and to lyse the cells. The release of intracellular CO2 was

captured by Whatman filter papers soaked in hyamine covering the lid over a period of 24 hours at room temperature. Next, the filter papers were transferred to scintillation vials and counted by the Scintillation Analyzer.

Oxidative Pentose Phosphate Pathway (oxPPP) was measured as $^{14}CO_2$ formation using [6-^{14}C]-D-glucose ($^{14}CO_2$ formation only in the TCA cycle) and [1-^{14}C]-D-glucose ($^{14}CO_2$ formation both in the TCA cycle and the oxPPP). Cortical neurons were cultured in parallel wells and incubated with medium containing [6-^{14}C]-D-glucose (1 μCi/mmol) or medium containing [1-^{14}C]-D-glucose (1 μCi/mmol). Released $^{14}CO_2$ was captured in a similar manner as described above for glucose oxidation. The [6-^{14}C]-glucose flux was then subtracted from the [1-^{14}C]-glucose flux. The relative contribution of the oxPPP flux to glucose metabolism was assessed by including glucose uptake in the relative flux measurements as described before (Aschroft et al 1972).

Glutamine oxidation was measured as $^{14}CO_2$ formation using [U-^{14}C]-glutamine. Cortical neurons were incubated with medium containing [U-^{14}C]-glutamine (as well as glucose). Similar as described above for glucose oxidation, cells were lysed with $HClO_4$ and $^{14}CO_2$ was captured in hyamine-soaked filter papers.

Lactate oxidation was measured in a similar way as $^{14}CO_2$ formation after incubation with glucose-, lactate- and glutamine-containing medium with [U-^{14}C]-lactate.

Oxygen consumption rate (OCR) was measured using the extracellular flux analyzer XF24 (Seahorse Bioscience Inc.). Cortical neurons were plated and grown for 7 days in a Seahorse XF24 tissue culture plate. At DIV7, the oxygen consumption rate was measured over a period of 2 minutes. In baseline conditions, 5 consecutive measurements of OCR are done. Next, oligomycin, a blocker of ATP synthase is injected at 12 μM (concentration in well 1.2 μM). The drop in OCR reflects the oxygen consumption serving ATP production. In parallel well dinitrophenol (DNP), a mitochondrial uncoupler is injected at 1 mM (concentration in well 100 μM). This will force neurons to use their

maximal mitochondrial capacity in order to preserve the mitochondrial membrane potential. The increase in OCR reflects the reserve capacity of mitochondria. As a second injection Antimycin A is added at 10 µM (concentration in well 1 µM), a complex III inhibitor. This shuts down the electron transport chain activity abolishing the mitochondrial oxygen consumption. The residual OCR corresponds to the non-mitochondrial oxygen consumption. All mitochondrial inhibitors are from Sigma.

10. ASSESSMENT OF REACTIVE OXYGEN SPECIES

The scavenging capacity of reactive oxygen species (ROS) was measured by assessing the detoxification rate of H_2O_2 after loading the cells with 10 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2-DCFDA, Molecular Probes, Invitrogen). CM-H_2-DCFDA is metabolized by intracellular esterases to a non-fluorescent product, which is oxidized to the fluorescent product CM-DCF, by H_2O_2. The dye was washed away and cells were exposed to 100 µM H_2O_2. Fluorescence was measured over time with a fluorescent plate reader.

11. MEASUREMENT OF ENERGY CHARGE

Energy charge was monitored over 8 hours of oxygen-nutrient deprivation. Cells were exposed for 8 hours to 0.1% oxygen in nutrient-deprived medium. Cells were harvested after 4, 6 and 8 hours in 100 µl ice cold 0.4 M perchloric acid supplemented with 0.5 mM EDTA. pH was adjusted by adding 100 µl of 2 M K_2CO3. 100 µl of the mixture was injected onto an Agilent 1260 HPLC equipped with a C18-Symmetry column (150 x 4.6 mm; 5 mm) (Waters), thermostated at 22.5 °C. Flow rate was kept constant at 1 ml/min. A linear gradient using solvent A (50 mM NaH_2PO4, 4 mM tetrabutylammonium, adjusted to pH 5.0 using H_2SO4) and solvent B (50 mM NaH_2PO4, 4 mM tetrabutylammonium, 30% CH_3CN, adjusted to pH 5.0 using H_2SO4) was accomplished as follows: 95% A for 2 min, from 2 to 25 min linear increase to

100% B, from 25 to 27 min isocratic at 100% B, from 27 to 29 min linear gradient to 95% A and finally from 29 to 35 min at 95% A. Detection of ATP, ADP and AMP occurred at 259 nm. The energy charge is calculated as ([ATP] + ½ [ADP] / [ATP] + [ADP] + [AMP])

12. IMMUNOCYTOCHEMISTRY

Cortical neurons grown on Willco-Dish glass bottom dishes (Ibidi) were after 7 days in culture fixed with PFA 4% for 10 minutes. After permeabilization for 10 minutes in 0.25% Triton and blocking 30 minutes with pre-immune serum, cells were incubated overnight with the appropriate primary antibodies. Following antibodies were used anti-TOMM20 (ab56783, Abcam; 1/200), anti-MAP2 (M3696, Sigma, 1/500), anti-GFAP (G3893, Sigma, 1/500), anti-NG2 (AB5320, Chemicon, 1/200), anti-Olig2 (AB9610, Chemicon, 1/20 000). Next day, sections were incubated with the appropriate fluorescently conjugated secondary antibody (Alexa-568 or Alexa 488, Molecular Probes, Invitrogen).

For Periodic Acid Schaff (PAS) staining, cells were exposed for 10 minutes to periodic acid, after which Shiff reagens (De Tomasi, Prosan NV) was added for 5 minutes. 10 µm thick cryofixed sections through embryonic brains from wild type and PHD1$^{-/-}$ embryos at E14.5-15.5 were stained with Nissl.

13. RNA AND DNA ANALYSIS

Total RNA was extracted from cultured cortical neurons using the RNeasy microkit (Qiagen), and reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Total RNA was extracted from dissected brain tissue using the Trizol-chloroform extraction. After tissue homogenization with Trizol, chloroform was added to separate the aqueous phase. After centrifugation and addition of ethanol, samples underwent a RNA clean-up on a RNeasy mini column (Qiagen 74104)

with DNAse I treatment (Invitrogen 12185010). Gene expression analysis was performed by Taqman quantitative RT-PCR, using premade primer sets (Applied Biosystems). Expression levels were quantified relative to the expression level of housekeeping genes (β-actin or HPRT).

For mitochondrial DNA copy measurement, total DNA was extracted from cell lysates. The amount of mitochondrial DNA relative to nuclear genomic DNA was determined by quantitative PCR using homemade primers for cytochrome b (mitochondrial) and RPL13A (nuclear): NC FOR2 agacctgaagaggcttcgctaa; NC REV2 cagcttgcgggtcaagct; NC probe2 ccaccgtcccttccagctaccatccta; MIT PROBE aca att agg gtt tac gac ctc gat gtt gga t; MIT FOR ttg atc aac gga cca agt tac c; MIT REV cgt tga aca aac gaa cca tta ata g.

14. PROTEIN ANALYSIS

Nuclear protein extraction was done by using the NEPER kit (Pierce, Thermo Scientific) according to manufacturer's instructions, in the presence of protease and phosphatase inhibitors (Roche). Lysates were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane (Life Technologies) and analyzed by immunoblotting. Primary antibodies used were goat anti-HIF-1α (AF1935, R&D Systems, 1:200), goat anti-HIF-2α (AF2997, R&D Systems, 1:200). Equal loading was verified by incubation with rabbit anti-βactin (13E5; No. 4970), rabbit anti-βtubulin (No. 2144), and rabbit anti-lamin A/C (No. 2032) (all from Cell Signaling Technology, Bioké). Appropriate secondary antibodies were from Dako (Enschede, the Netherlands). Signal was detected using the ECL system (Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions.

15. INTRACEREBROVENTRICULAR DELIVERY OF ANTISENSE OLIGONUCLEOTIDES AGAINST PHD1 IN STROKE

Oligonucleotides directed against murine PHD1 were synthesized and purified by Isis Pharmaceuticals as previously described (Bennett and Swayze 2010). These antisense oligonucleotides (ASOs) are directed against the regions within the coding region and 5' and 3' untranslated region of the PHD1 mRNA. The principle of knockdown by these ASOs is that after binding to complementary target RNA through Watson-Crick base-pairing, the target RNA will be degraded by RNaseH-mediated RNA degradation. Additional modifications consisting of a *O*-(2-methoxy)ethyl substitution at the 2' position and phosphorothioate modifications were created to increase the resistance of the ASOs against nuclease activity and in this way promote their stability. Three top ASOs of 20 nucleotides long were selected for further investigation.

Knockdown efficiency was tested in cultured cortical neurons. At DIV2, cortical neurons were exposed to 0.5 µM and 1 µM of anti-PHD1 ASOs. To ensure correct dosing, the absorbance at 260 nm was regularly measured in the stock solution. An oligonucleotide that targets murine MALAT-1 (non coding RNA in the nucleus) was used as control ASO. According to the experience of Isis Pharmaceuticals, this oligonucleotide does not induce a phenotype in the central nervous system and is well tolerated in neurons. At DIV7, cortical neurons were harvested for RNA extraction and qPCR for PHD1, PHD2 and PHD3.

One out of the three ASOs with the highest efficiency and specificity was selected for further *in vivo* studies. A dose of 75 µg ASO/day was infused into the left lateral ventricle of male C57/Bl6 mice (age 7-8 weeks), using Alzet® osmotic pumps (model 2004, Cupertino), connected with a catheter to a brain infusion cannula as previously described (Storkebaum et al 2005). The brain infusion assembly was filled with a solution, containing the ASO diluted in artificial CSF (aCSF) or with aCSF alone (saline control). For the *in vivo* studies both saline and a non-RNAas H active ASO to human huntingtin was used as control. The composition of the aCSF was 150 mM Na^+, 3 mM K^+, 1.4

mM Ca^{2+}, 0.8 mM Mg^{2+}, 1 mM PO_4^{3-} and 155 mM Cl^-. After anesthesia with ketamine and xylazine, the osmotic pump was subcutaneously inserted in the midscapular area on the back of the mouse. After exposing the skull through a midline sagittal incision, a hole was drilled through the skull, and the cannula placed using the following stereotactic coordinates: 0 mm posterior and 1 mm lateral to bregma, and 2 mm deep from skull surface. After 10 days of infusion, brains were harvested from a series of test mice to assess knockdown efficiency. Two weeks after the infusion, pMCAO was performed

16. ROTAROD AND SURVIVAL ANALYSIS IN SOD1^{G93A} MICE

Female PHD1$^{+/+}$ and PHD1$^{-/-}$ x SOD1^{G93A} littermates were followed up twice a week by an investigator who was blinded to the genotype. Motor performance was tested by the use of the rotarod (Ugo Basile) twice a week from the age of 60 days. Mice were tested for their ability to stay on the rotarod, which has a constant rotating speed of 15 rotations per minute, for 3 minutes. Time of onset of paralysis was determined as the time when mice fell of the rotarod before 1 minute (on average in 5 separate trials). Body weight was also followed up twice a week. Time of death was scored as the day when the mice had lost 20% of their pre-symptomatic body weight or when they were no longer able to right themselves within 30 seconds when placed on their back.

17. STATISTICS

All data are reported as mean ± SEM of the indicated number of experiments or mice. For *in vitro* data the N-values indicate the number of independent experiments performed with different cultures of WT and PHD1$^{-/-}$ cortical neurons. When inter-experimental variability was too large, representative experiments are shown (with technical replicates; as indicated). In the shown representative experiment the differences between the conditions reflects the average of the pooled experiments. In that case, at least 3 independent

experiments were performed each showing statistical significance ($p<0.05$) using the appropriate statistical test. Student's t-test was used unless another statistical test is mentioned.

In some $PHD1^{-/-}$ mice no infarct was visible after ligation of the middle cerebral artery (indicating complete protection against brain ischemia), thereby disturbing normal Gaussian distribution. In these cases, we used non-parametrical Mann-Whitney-U test to determine statistical differences between the two groups. Kaplan-Meier statistics were used for analysis of rotarod performance and survival in $SOD1^{G93A}$ mice. We used Prism v5.0 for all statistical calculations. $P<0.05$ was considered statistically significant.

Chapter IV

RESULTS

PHD1$^{-/-}$ MICE ARE PROTECTED AGAINST ISCHEMIC STROKE AFTER PERMANENT MCAO

Ischemic stroke is the most striking and acute example of a collapsing oxygen homeostasis in the brain. We hypothesized that deletion of one of the PHD isoforms would increase the brain's resilience against ischemia. To address the role of the different PHD isoforms, we induced acute ischemic stroke in different PHD-deficient mice. Through a permanent ligation of the middle cerebral artery, distal to the lenticulostriate arteries, acute ischemia in the sensorimotor cortex was induced. We chose this distal model of permanent middle cerebral artery occlusion (pMCAO) because it is known for its high reproducibility and low mortality rates (Kuraoka et al 2009). Since we earlier reported that mice lacking PHD1 are protected against limb and liver ischemia (Aragones et al 2008, Schneider et al 2009), we initiated our stroke studies in homozygous PHD1 deficient (PHD1$^{-/-}$) mice.

We performed pMCAO in PHD1$^{-/-}$ mice and wild type males (C57/Bl6 background), aged 8-11 weeks. To assess the infarction area, we stained brain slices with the vital dye TTC (2,3,5-triphenyltetrazolium chloride) at 24 hours post-pMCAO (when lesion size after pMCAO is maximal (Kuraoka et al 2009)). This analysis revealed that brain infarction is largely attenuated in PHD1$^{-/-}$ mice with a ca. 70% reduction in ischemic lesion size in PHD1$^{-/-}$ mice *versus* control wild type mice (Figure 1A-C).

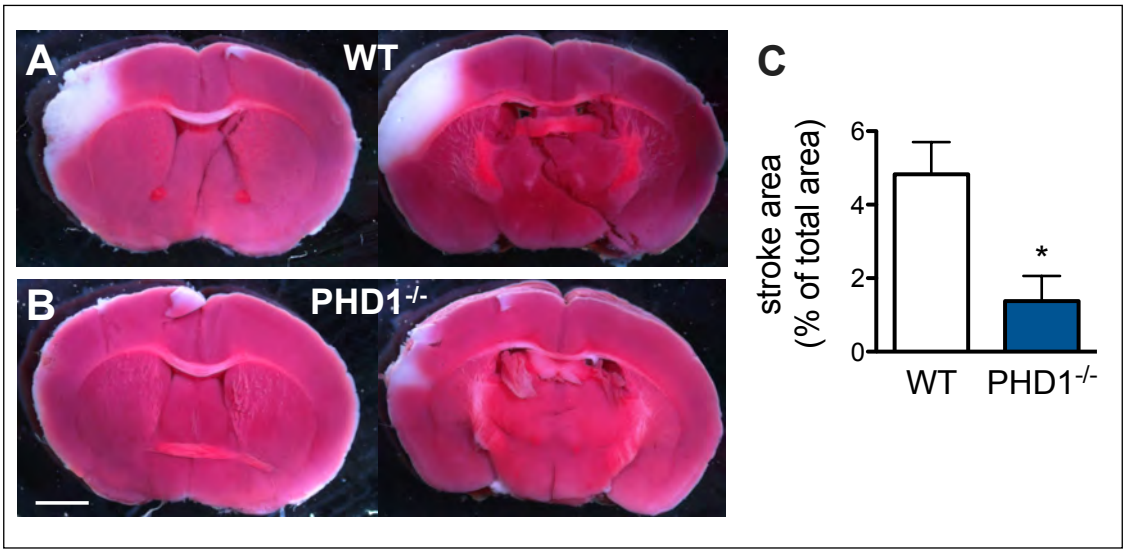

Figure 1: PHD1 deficiency reduces infarct size after pMCAO (C57/Bl6 strain)
Representative brain slices after TTC staining, delineating the infarct zone as unstained area (white), from wild type (WT) (A) and PHD1$^{-/-}$ mice (B) 24 hours after pMCAO. Quantification of the stroke area as percentage of the total bihemispheric area is shown in C (N=6, *P<0.05, Mann-Whitney U test). Scale bar: 1 mm.

To test whether this smaller infarct size also resulted in improved functional outcome, we used the adhesive tape removal test (Bouet et al 2009). This test was specifically developed as a very sensitive assessment of sensory and motor coordination in this distal model of pMCAO, where more classic motor tests such as grip strength and rotarod performance are unaffected. In wild type mice, as expected, an important delay in the time needed to sense the tape on their affected forepaw as well as in the time necessary to remove the tape arose after ischemia-induction (Figure 2A,B). In contrast, post-stroke functional performance in PHD1$^{-/-}$ mice was hardly affected (Figure 2A,B). Of importance, this functional difference between wild type and PHD1$^{-/-}$ mice persisted over a longer period of time (Figure 2A,B), indicating that the protective effect in the PHD1$^{-/-}$ mice was not a temporary effect, only observable at 24 hours after the ligation.

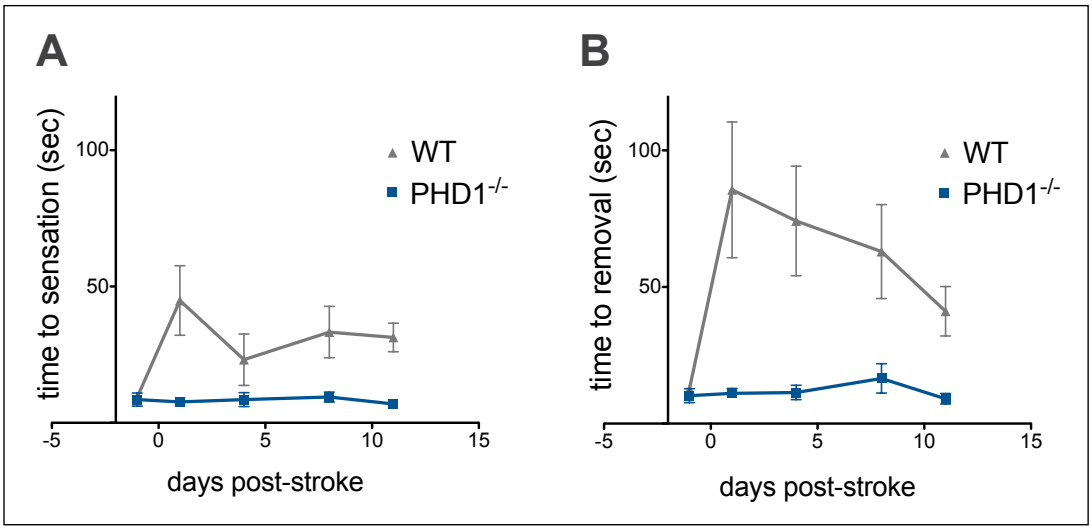

Figure 2: PHD1 deficiency improves functional performance in the adhesive tape removal test after stroke induction
(A) Average time needed before the WT (grey) and PHD1$^{-/-}$ (blue) mice sense the presence of the tape, assessed 1 day before and at day 1, 4, 8 and 11 post-stroke (N=7-8, **P<0.01, two-way ANOVA). (B) Average time needed before the mice successfully removes the tape, assessed 1 day before and at day 1, 4, 8 and 11 post-stroke (N=7-8, **P<0.01, two-way ANOVA).

Differences in stroke size depending on the mouse strain used have been extensively reported in the literature (Barone et al 1993, Connolly et al 1996, Fujii et al 1997, Majid et al 2000). Differences in vascular anatomy (e.g. completeness of circle of Willis), vascular responses to anesthetics, neuron-intrinsic resilience and many other factors were described to underlie this strain-specific vulnerability (Beckmann 2000, Fujii et al 1997). Therefore, to exclude strain-specific protection, we also performed pMCAO also in the 129S6 strain. The average stroke size in wild type mice was smaller in the 129S6 than in the C57/Bl6 strain. Still, PHD1$^{-/-}$ brains were protected against ischemic injury to a similar extent as shown by TTC staining (Figure 3A-C).

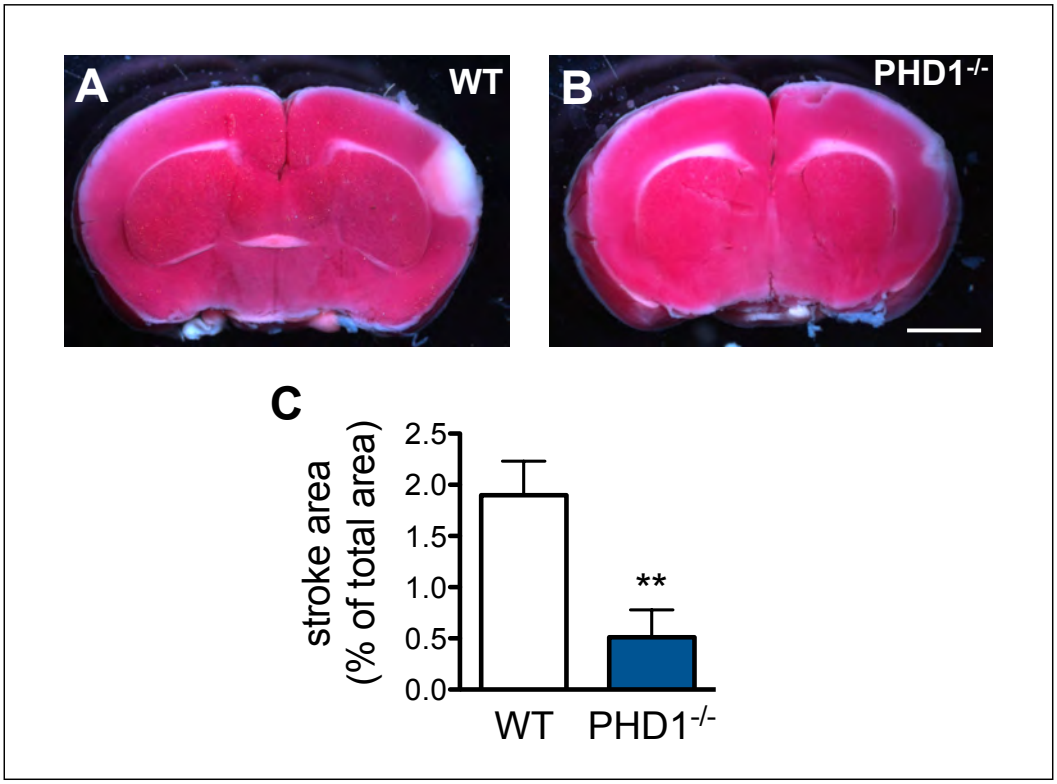

Figure 3: PHD1 deficiency reduces infarct size after pMCAO (129S6 strain).
Representative brain slices after TTC staining, delineating the infarct zone as unstained area (white), from WT (A) and PHD1$^{-/-}$ mice (B) 24 hours after pMCAO. Quantification of the stroke area as percentage of the total bihemispheric area is shown in C (N=6-9, **P<0.01, Mann-Whitney U test). Scale bar: 1 mm.

To address the specificity of this ischemia tolerance for PHD1, we also assessed the effect of PHD2 and PHD3 deficiency on stroke size. As homozygous PHD2 deficiency causes embryonic lethality (Takeda et al 2006), we induced a neural-specific PHD2 deletion by intercrossing PHD2$^{lox/lox}$ mice with Nestin-Cre mice (Tronche et al 1999). Infarct size was not affected in Nestin-Cre^{+} x PHD2$^{lox/lox}$ mice when compared to Nestin-Cre^{-} x PHD2$^{lox/lox}$ mice (Figure 4A-C). Also no differences in stroke size could be observed in PHD3$^{-/-}$ mice as compared to wild type mice (Figure 4D-F). In addition, PHD1$^{-/-}$ brains did not have augmented transcript levels of the other oxygen sensors PHD2 and PHD3 (relative mRNA levels in PHD1$^{-/-}$ brain compared to WT brain: 106.2

± 5.0% for PHD2 and 105.7 ± 2.2% for PHD3; N=4; P=0.34 for PHD2 mRNA and P=0.35 for PHD3 mRNA). This suggests that constitutive PHD1 deficiency does not elicit compensatory changes in the other oxygen sensors, further substantiating a PHD1-specific neuroprotective effect in stroke.

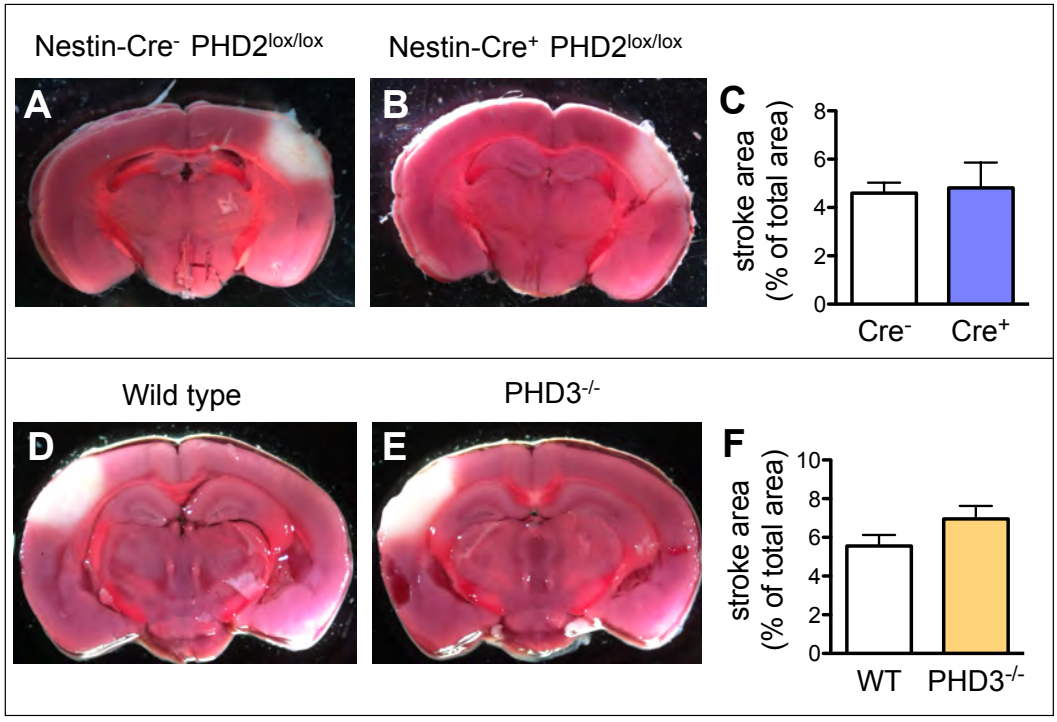

Figure 4: Deficiency of PHD2 or PHD3 does not affect infarct size after pMCAO.
Representative images from TTC staining in Nestin-Cre^{-} PHD2$^{lox/lox}$ *versus* Nestin-Cre^{+} PHD2$^{lox/lox}$ (A,B) and WT *versus* PHD3$^{-/-}$ mice (D,E). Quantification of the stroke area as percentage of the total bihemispheric area is shown in (C) for Nestin-Cre^{-} PHD2$^{lox/lox}$ *versus* Nestin-Cre^{+} PHD2$^{lox/lox}$ (N=3; P=0.86) and in (F) for WT *versus* PHD3$^{-/-}$ mice (N=7-8; P=0.14).

PHD1$^{-/-}$ BRAIN VASCULATURE DOES NOT SHOW MAJOR MORPHOLOGICAL OR FUNCTIONAL ALTERATIONS

Angiogenesis is a hypoxia-driven process (Carmeliet and Jain 2011, Quaegebeur et al 2011). PHDs have been described to drive angiogenesis and regulate perfusion in different organs as a means to increase oxygen delivery (Quaegebeur and Carmeliet 2010, Takeda et al 2011). Also, collateral flow and hence residual vascular perfusion determines stroke size both in mice and in patients (Shuaib et al 2011, Zhang et al 2010). Therefore, we explored whether PHD1 deficiency would protect against ischemia by affecting the brain vasculature and improving cerebral perfusion.

Perfusion with FITC-conjugated dextran (unable to leak out of brain vessels given its molecular weight of 20,000 kDa) was used to assess the patency of cerebral vessels. Quantification showed a similar area of perfused vessels in the cortical area of (non-stroked) wild type *versus* PHD1$^{-/-}$ brains (Figure 5A-C). To explore whether PHD1 deficiency affects arteriogenesis in the brain, we stained (non-stroked) wild type and PHD1$^{-/-}$ brains for α-smooth muscle actin (α-SMA), visualizing the larger conducting arterioles and arteries. This analysis did not reveal genotypic differences either (Figure 5D-F).

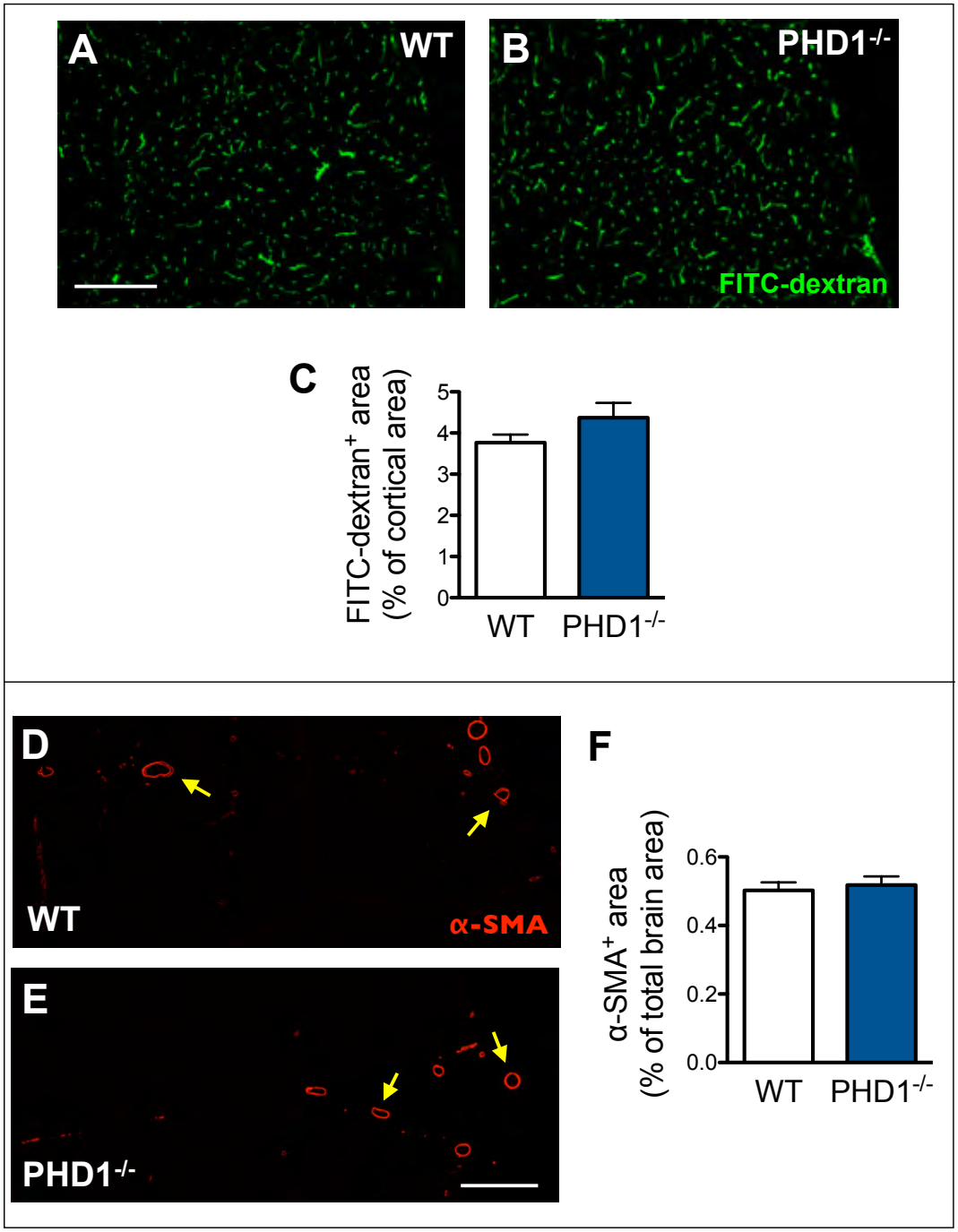

Figure 5: The PHD1$^{-/-}$ brain does not show more vessel perfusion or more arterioles

Representative images of WT (A) and PHD1$^{-/-}$ (B) frontal cortex after FITC-dextran injection. Quantification of the FITC-dextran-positive area as percentage of the cortical area (reflecting vessel perfusion in the cortex) is shown in C (N=6-7, P=0.19). Representative images of α-SMA immunostaining, visualizing arterioles (yellow arrows), in WT (D) and PHD1$^{-/-}$ brains (E). Quantification of α-SMA positive area as percentage of total brain area is shown in F (N=4-5, P=0.67). Scale bar A,B: 200 µm; D,E: 500 µm.

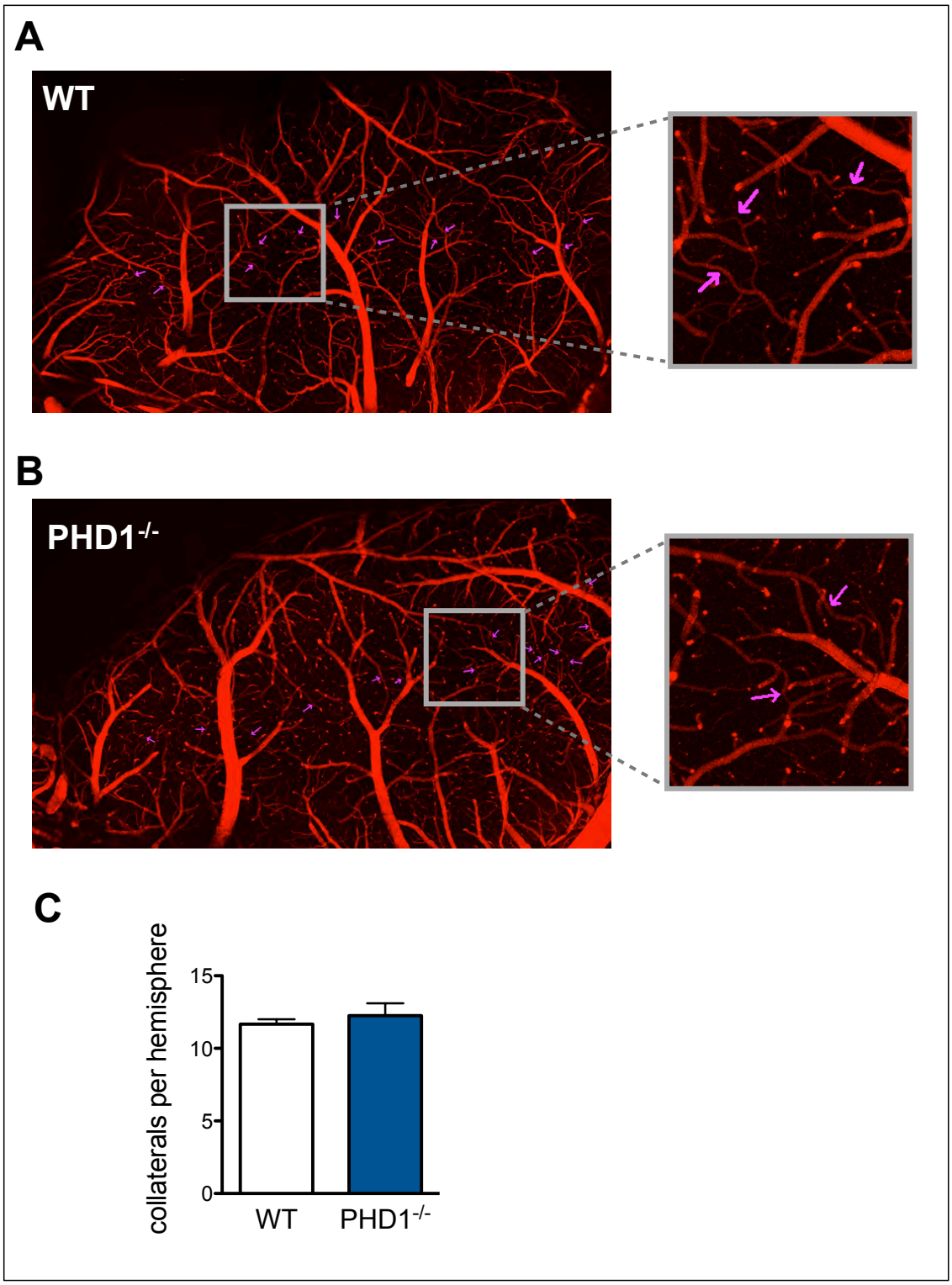

Figure 6: The PHD1$^{-/-}$ brain does not show enhanced pial collateral circulation

(A-B) Representative images of 3D vascular corrosion casting showing pial circulation in WT (A) and PHD1$^{-/-}$ brain hemispheres (B). Collaterals were counted per hemisphere (purple arrows). (C) Quantification of pial collaterals per hemisphere in WT *versus* PHD1$^{-/-}$ brains (N=3-4; P=0.60)

Since arteriogenesis is unaffected, it would seem less probable that the stroke area in the PHD1$^{-/-}$ brain receives more collateral blood flow at the time of the arterial occlusion. To exclude this with more certainty, we specifically imaged the pial collateral circulation, as these collaterals are known to contribute to collateral flow in the stroke area following pMCAO (Zhang et al 2010). After infusion of a resin, a three dimensional corrosion cast of the brain vasculature was made. We quantified the number of pial collaterals in one hemisphere, which mainly reflect the bridging arterioles between the anterior cerebral artery and the middle cerebral artery (Zhang et al 2010). This showed a similar number of pial collaterals in wild type and PHD1$^{-/-}$ brain (Figure 6A-C).

These data indicate that PHD1 deficiency does not majorly affect the vasculature in the brain. Systemic erythropoietin (EPO) and hematocrit levels, which determine the oxygen carrying potential of the blood and might affect the infarct size by improving tissue oxygenation (Sakanaka et al 1998, Siren et al 2001), were already shown to be unaffected in PHD1$^{-/-}$ mice in a previous report (Aragones et al 2008). In conclusion, PHD1 deficiency does not protect against brain ischemia via improving oxygen delivery.

PHD1$^{-/-}$ NEURONS ARE PROTECTED AGAINST OXYGEN-NUTRIENT DEPRIVATION IN VITRO

Given the lack of clear vascular changes in the PHD1$^{-/-}$ brain, we next hypothesized that PHD1 deficiency would confer neuroprotection by modulating intrinsic neuronal properties. We therefore assessed the effect of ischemia in cultured cortical neurons. We exposed cortical neurons (isolated from wild type and PHD1$^{-/-}$ E14.5-15.5 embryonic brains) after 7 days in culture to oxygen-nutrient deprivation for a 2 hour period. At this time, the cortical neurons have evolved into fully mature neurons, carrying features of adult neurons (Dotti et al 1988). In this period they are kept at 0.1% oxygen in medium lacking not only glucose but also glutamine and pyruvate, after which they were re-exposed to ambient air and nutrient-rich medium. This assay, often incorrectly referred to as oxygen-glucose deprivation (as it is a more global nutrient deprivation), has been extensively used in the literature as an *in vitro* model of ischemia to test the intrinsic protective properties of neurons against ischemia (Goldberg and Choi 1993). During the oxygen-nutrient deprivation period, energy homeostasis will be acutely challenged. A small fraction of neurons will undergo immediate necrotic death due to severe ATP depletion, whereas the majority will undergo a delayed apoptotic death (Meloni et al 2011). The latter event will even be further facilitated by the re-exposure to oxygen and nutrients, which causes a surge in oxygen radicals, resulting in the so-called “reperfusion injury”.

Cell death was measured 24 hours after the oxygen-nutrient deprivation injury by quantifying LDH release in the medium, a method often used to assess neuronal cell death in culture (Koh and Choi 1987). Whereas 40% cell death was observed in wild type neurons, cell death was largely prevented in PHD1$^{-/-}$ neurons (Figure 7), almost approaching baseline cell death over 24 hours in the control normoxia condition, which was around 15% and similar between the two genotypes (not shown).

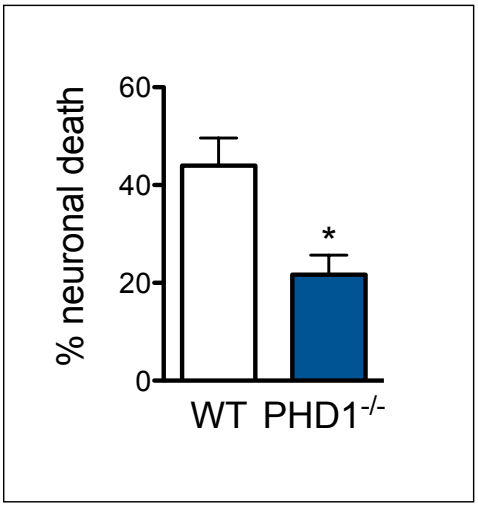

Figure 7: PHD1$^{-/-}$ neurons are largely protected against oxygen-nutrient deprivation.
Neuronal cell death measured as LDH release (% of maximal LDH release) over 24 hours after oxygen-nutrient deprivation in WT and PHD1$^{-/-}$ neurons (N=3, *P<0.05).

To exclude that structural differences in the PHD1$^{-/-}$ embryos or different PHD1$^{-/-}$ neuronal culture features would contribute to this resilience against *in vitro* ischemia, we performed additional control experiments. Macroscopic inspection of embryos revealed that loss of PHD1 did not cause prominent developmental central nervous system defects. Nissl staining revealed a normal brain cytoarchitecture in PHD1$^{-/-}$ embryos (data not shown). In agreement, isolation from E14.5 PHD1$^{-/-}$ embryos yielded similar numbers of cortical neurons. These appeared morphologically normal, showed similar neurite arborization, established comparable numbers of synapses, and survived for normal periods comparable to wild type neurons (data not shown). There were also no genotypic differences in the fraction of contaminating non-neuronal cells (< 2%, determined by staining for GFAP, NG2 and Olig2).

In order to exclude chronic compensatory changes due to constitutive absence of PHD1, we measured the transcript levels of PHD2, PHD3 or FIH in PHD1$^{-/-}$ neurons, which were no different than in their wild type counterparts (Figure 8A). These data are in accordance with the absence of induced expression of other PHD isoforms in PHD1$^{-/-}$ brains. Of importance, PHD2 and

PHD3 silencing (by means of lentiviral shRNA) did not confer protection against oxygen-nutrient deprivation, indicating a PHD1-specific effect, again in accordance with the PHD1 specific protection in the pMCAO model (Figure 8B).

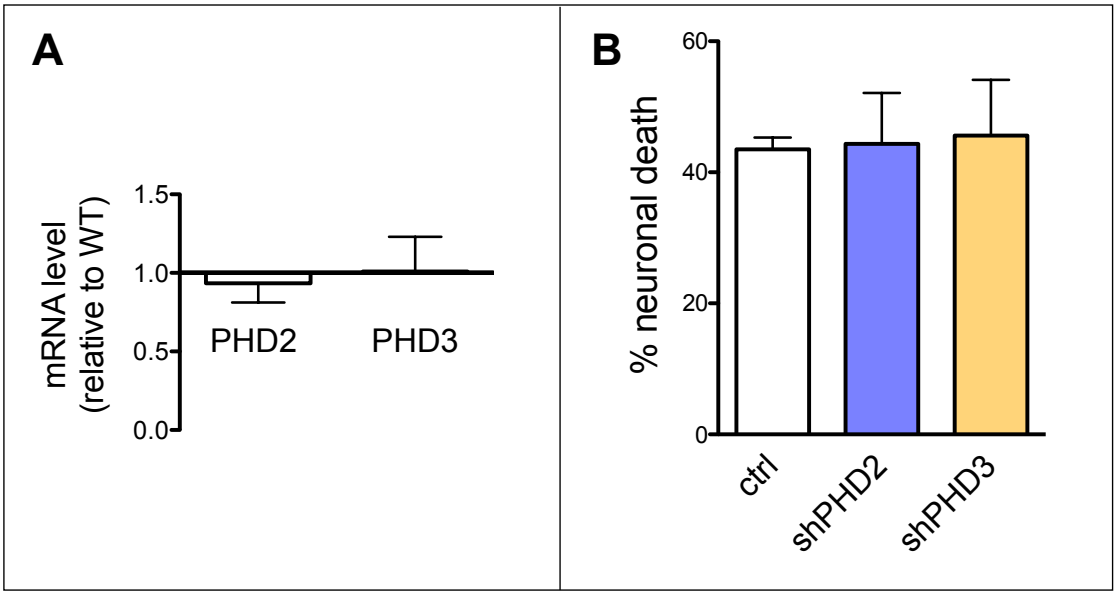

Figure 8: The protective effect against oxygen-nutrient deprivation is PHD1-specific.
(A) mRNA levels of PHD2 and PHD3 in isolated PHD1$^{-/-}$ neurons expressed as fold change relative to WT (N=3, P=0.66 for PHD2 mRNA, P=0.98 for PHD3 mRNA). (B) Neuronal cell death measured as LDH release (% of maximal LDH release) over 24 hours after oxygen-nutrient deprivation in PHD2 silenced and PHD3 silenced cells compared to control (N=3, P=0.92 for shPHD2 vs ctrl), P=0.83 for shPHD3 vs ctrl).

It has been established for a while that enhanced neurotrophin expression protects against a variety of stressors, among which ischemia (Ferenz et al 2012, Klumpp et al 2006). Therefore, we quantified the expression of different neurotrophic factors and their receptors in cultured neurons. No statistical significant differences were observed between wild type and PHD1$^{-/-}$ neurons (table 1)

Table 1: Gene expression analysis of neurotrophic factors

GENE	WT NEURONS	PHD1$^{-/-}$ NEURONS
Bdnf	1.00 ± 0.12	0.87 ± 0.07
Ngf	1.00 ± 0.12	0.88 ± 0.09
Nt3	1.00 ± 0.14	0.93 ± 0.08
p75	1.00 ± 0.09	0.87 ± 0.14
TrkB	1.00 ± 0.08	0.97 ± 0.10
TrkC	1.00 ± 0.14	0.95 ± 0.20
Vegf-a	1.00 ± 0.11	1.11 ± 0.06
Vegf-b	1.00 ± 0.03	0.88 ± 0.09
Vegfr2	1.00 ± 0.15	0.81 ± 0.06

Quantitative RT-PCR data of different neurotrophic factors and their receptors in WT and PHD1$^{-/-}$ neurons. Representative experiments of fold expression in the PHD1$^{-/-}$ neurons compared to WT neurons are shown (mean±SEM, N=3-4, P=ns). Bdnf: Brain-derived neurotrophic factor; Ngf: Nerve growth factor; Nt3: Neurotrophin 3; p75: p75 neurotrophin receptor; TrkB and TrkC: tyrosine receptor kinase B and C; Vegf-a and Vegf-b: vascular endothelial growth factor A and B; Vegfr2: Vascular endothelial growth factor 2.

PHD1 DEFICIENCY REPROGRAMS GLUCOSE METABOLISM IN NEURONS

PHD1 deficiency has previously been shown to protect against ischemia in the muscle via altering its glucose metabolism (Aragones et al 2008). By reducing pyruvate entry into the mitochondria, $PHD1^{-/-}$ muscle fibers reduce their respiration rate and, as a consequence, produce less reactive oxygen species (ROS). In addition, glycolytic flux is increased, likely as a compensation to maintain sufficient ATP production. Hence, we next investigated whether a similar metabolic shift would explain the ischemia tolerance in $PHD1^{-/-}$ neurons. To explore glucose metabolism, we measured metabolic fluxes by incubating cultured neurons for 2 hours with a radioactively-labeled glucose tracer. Postulating that metabolic changes might account for the protective effect, all metabolic measurements were performed after neurons spent 7 days in culture, the same time that cultures are subjected to oxygen-nutrient deprivation.

In sharp contrast to the metabolic phenotype of $PHD1^{-/-}$ muscle fibers, the glycolytic rate of $PHD1^{-/-}$ neurons, quantified by the production of $^{3}H_2O$ after supplementation with [5-^{3}H]-glucose, was reduced – not increased – by 25.4 ± 4.2% (nmol glucose/h/10^6 cells: 285 ± 85 for wild type *versus* 199 ± 54 for $PHD1^{-/-}$ neurons, N=8, *P<0.05, Figure 9A,B). Measuring $^{14}CO_2$ release after incubation with [6-^{14}C]-glucose showed a 23 ± 3.4% reduction in the glucose oxidation rate in $PHD1^{-/-}$ neurons (pmol glucose/h/10^6 cells: 1425 ± 176 for wild type *versus* 1116 ± 179 for $PHD1^{-/-}$ neurons, N=4, ***P<0.001, Figure 9C,D). To discriminate whether this was due to a reduced entry of glycolytic intermediates (as a result from the reduction in glycolytic flux) or to an impaired mitochondrial activity, we also supplied [U-^{14}C]-lactate, which can be converted to [U-^{14}C]-pyruvate, enter the TCA cycle and become oxidized in the same way as [6-^{14}C]-glucose. These measurements showed that [U-^{14}C]-lactate oxidation was similar for both genotypes (pmol lactate/h/10^6 cells: 6327 ± 178 for wild type *versus* 6564 ± 596 for $PHD1^{-/-}$ neurons; N=3; P=0.7), indicating that a reduced entry of glycolytic intermediates rather than a mitochondrial defect caused the reduced glucose oxidation.

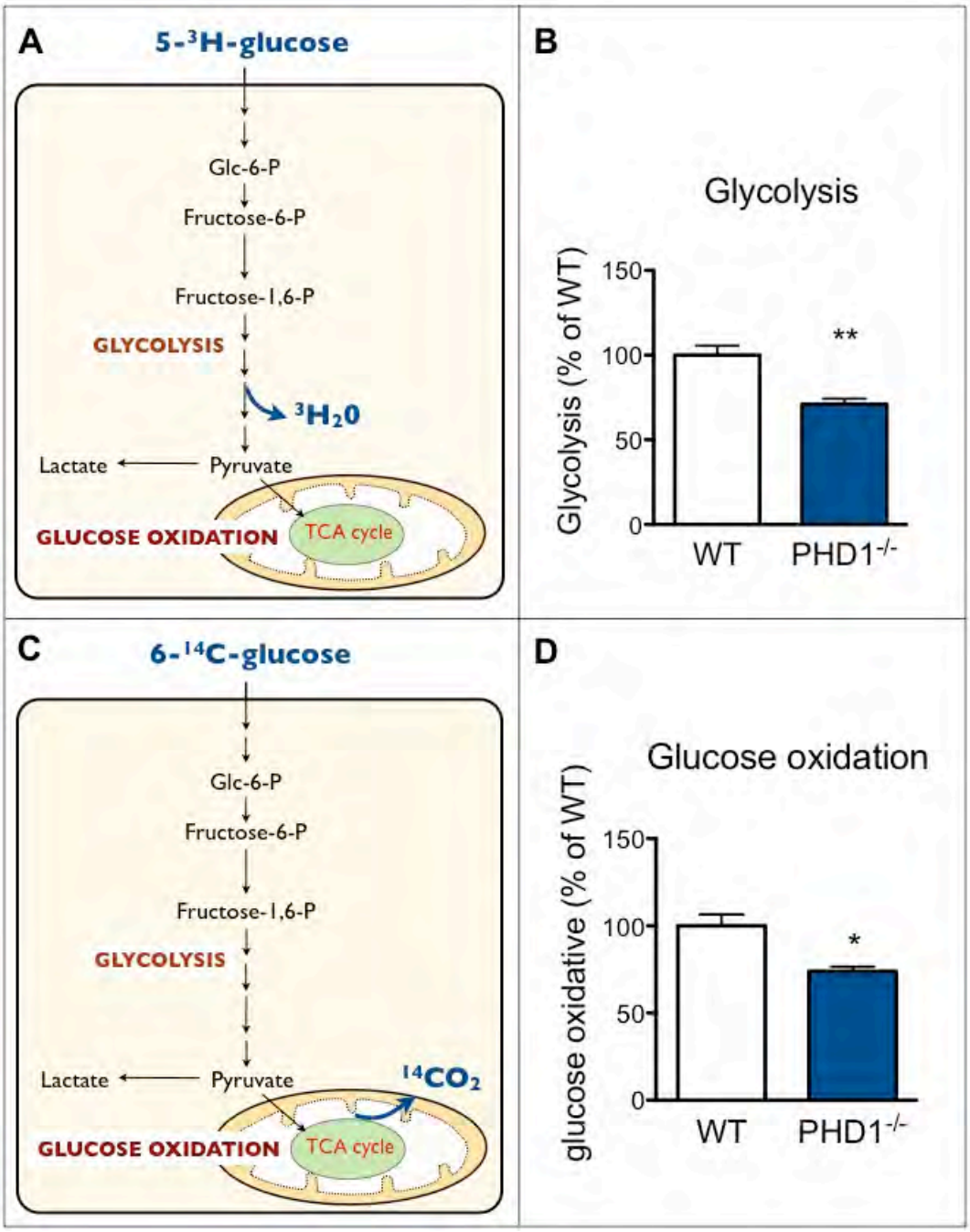

Figure 9 PHD1$^{-/-}$ neurons reduce their glycolytic flux and glucose oxidation rate.

(A) Schematic overview of glycolytic flux measurements via incubation of cells with tracer amounts of 5-^{3}H-glucose. The resulting $^{3}H_2O$ release during glycolysis reflects the rate of glycolysis. (B) Representative experiment of relative (%) change of glycolytic flux measured over 2 hrs in PHD1$^{-/-}$ neurons compared to WT neurons (N=4, **P<0.01). (C) Schematic overview of glucose oxidation rate measurements via incubation of cells with tracer amounts of 6-^{14}C-glucose. $^{14}CO_2$ will be released through oxidation of TCA intermediates in the TCA cycle, reflecting the rate of glucose oxidation. (D) Representative experiment of relative (%) change of glucose oxidation measured over 2 hrs in PHD1$^{-/-}$ neurons compared to WT neurons (N=3, *P<0.05).

The reduced glucose oxidation was also not due to a decrease in the number of mitochondria as determined by PCR analysis of mitochondrial (mtDNA) versus genomic (gDNA) DNA (ratio gDNA/mitoDNA: 1.595 ± 0.065 for WT *versus* 1.565 ± 0.032 for $PHD1^{-/-}$ neurons; N=3; P=0.7). In line with this, mitochondrial density as analyzed by staining of neurons for the mitochondrial marker TOMM20 revealed no genotypic differences (Figure 10A-G).

Also at the functional level, $PHD1^{-/-}$ mitochondria behaved similarly as their WT counterparts: the oxygen consumption rate (OCR), measured using the Seahorse extracellular flux analyzer, was not altered in $PHD1^{-/-}$ neurons (Figure 11B) (this apparent paradox with the reduced glucose oxidation rate will be explained below). Besides baseline OCR (OCR_{BAS}), we also measured OCR coupled to ATP synthesis (OCR_{ATP}; sensitive to oligomycin) and maximal respiration (OCR_{MAX}; induced by the uncoupler DNP) (Figure 11A). PHD1 deficiency did not affect these bioenergetic features of the mitochondria either (Figure 11C).

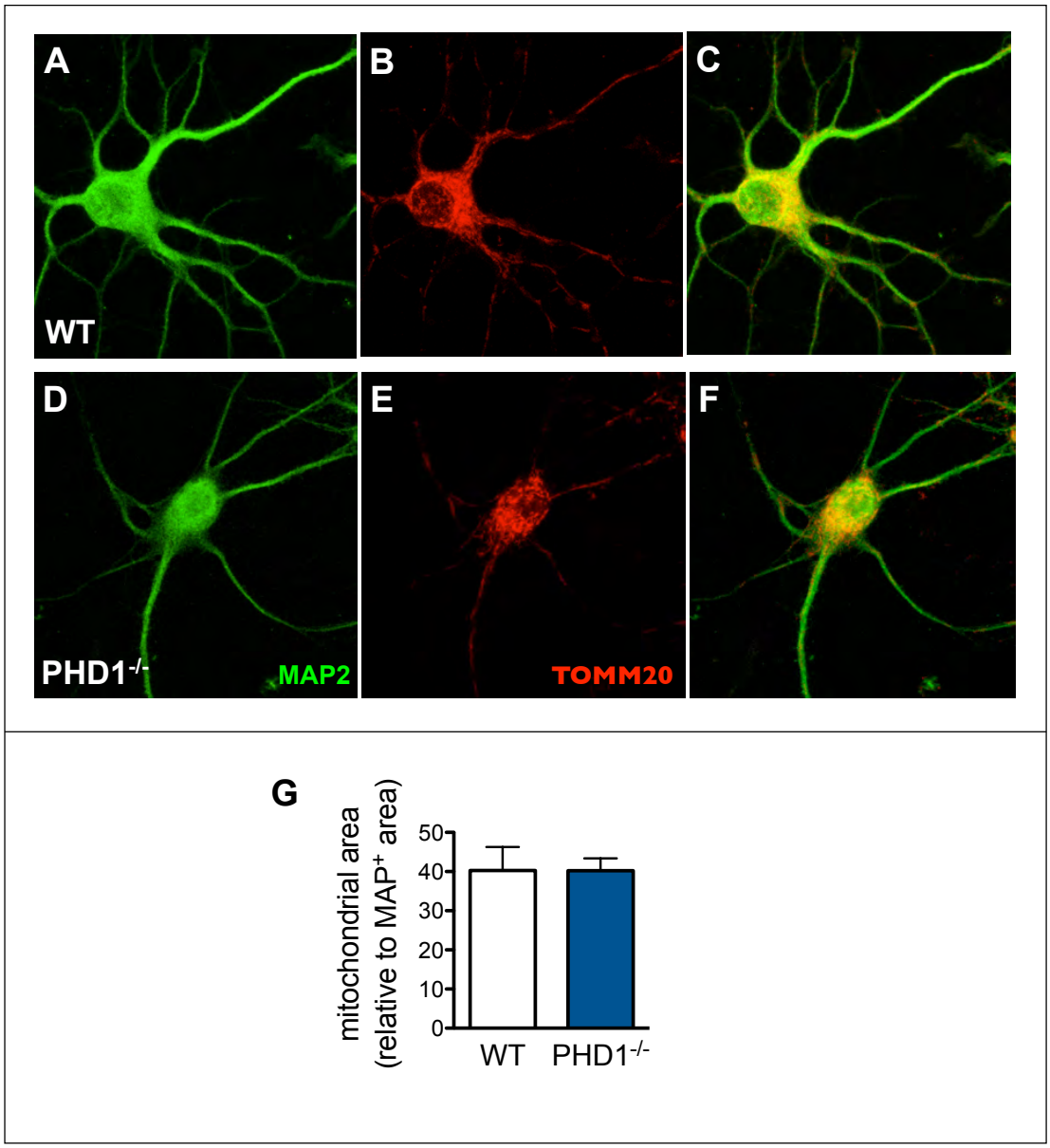

Figure 10: PDH1$^{-/-}$ neurons show a similar number of mitochondria
(A-F) Representative images from TOMM20 and MAP2 immunostaining, visualizing mitochondria and neurons respectively, in WT (A-C) and PHD1$^{-/-}$ neurons (D-F). Quantification of mitochondrial area per cell (i.e. MAP2 positive area) is shown in G (N=3, P=0.87).

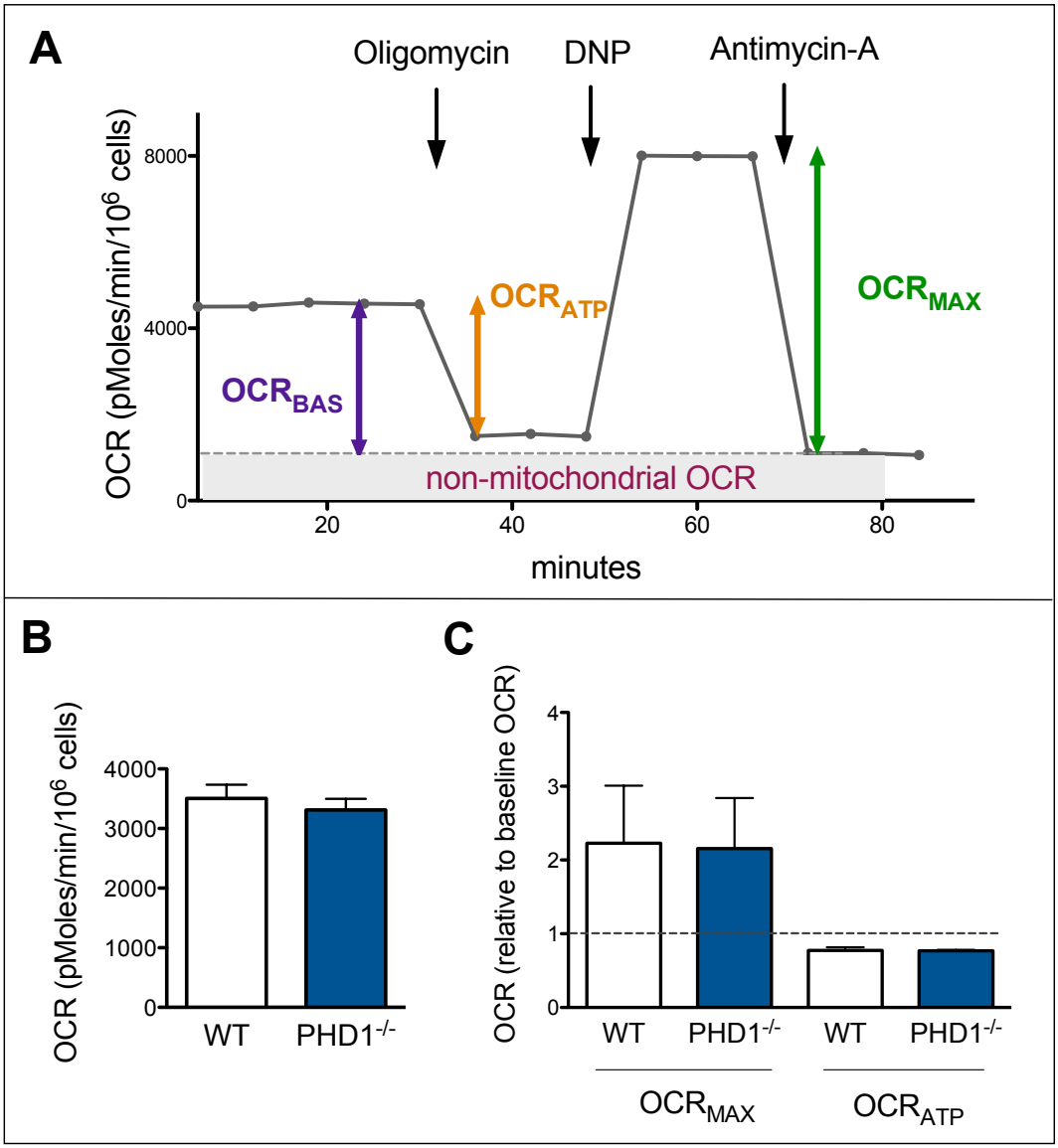

Figure 11: PHD1$^{-/-}$ mitochondria show similar bioenergetics features

(A) The Seahorse extracellular flux analyzer allows the measurement of different bioenergetics parameters by sequential injection of different mitochondrial compounds. Antimycin A injection blocks complex III and allows the calculation of baseline mitochondrial respiration (OCR_{BAS}). Oligomycin injection blocks ATP synthase and allows the measurement of respiration coupled to ATP synthesis (OCR_{ATP}). Maximal respiration (OCR_{MAX}) is induced by the uncoupler DNP. (B) Representative experiment of OCR_{BAS} in WT and PHD1$^{-/-}$ neurons (N=8-9; P=0.5). (C) Relative change of OCR_{ATP} and OCR_{MAX} compared to OCR_{BAS} (dotted line) in WT and PHD1$^{-/-}$ neurons (N=3; P=0.65 for OCR_{MAX} and P=0.94 for OCR_{ATP}).

Overall, in sharp contrast with the metabolic phenotype in the PHD1 deficient muscle, PHD1$^{-/-}$ neurons do not show a shift from oxidative to anaerobic metabolism, but maintain their mitochondrial metabolism and even lower their glycolytic flux. Notably, gene expression analysis could not link the reduced glycolysis to transcriptional changes in metabolic enzyme expression (table 2).

Table 2: Gene expression analysis of glycolytic enzymes

GENE	WT NEURONS	PHD1$^{-/-}$ NEURONS
Glut-1	1.00 ± 0.02	0.92 ± 0.05
Glut-3	1.00 ± 0.20	0.93 ± 0.05
Hk1	1.00 ± 0.05	1.11 ± 0.08
Pfk1	1.00 ± 0.20	0.80 ± 0.09
Pfkfb3	1.00 ± 0.09	1.02 ± 0.10
Gapdh	1.00 ± 0.09	0.92 ± 0.09
Pgam2	1.00 ± 0.20	1.30 ± 0.26
enolase	1.00 ± 0.07	1.24 ± 0.11
Ldh-a	1.00 ± 0.06	0.93 ± 0.13
Pdk1	1.00 ± 0.10	0.95 ± 0.08
Pdk4	1.00 ± 0.03	1.07 ± 0.04

Quantitative RT-PCR data of different glycolytic genes in WT and PHD1$^{-/-}$ neurons in baseline conditions. Representative experiments of fold expression in the PHD1$^{-/-}$ neurons compared to WT neurons are shown (mean± SEM, n=3-4, p=ns). Glut1: glucose transporter 1; Hk1: hexokinase; Pfk1: phosphofructokinase; Pfkfb3: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; Gapdh: glyceraldehyde-3-phosphate dehydrogenase; Pgam2: phosphoglycerate mutase 2; Ldh-a: lactate dehydrogenase a; Pdk1: pyruvate dehydrogenase kinase, isoform 1.

PHD1$^{-/-}$ NEURONS SHOW AN ENHANCED FLUX THROUGH THE OXPPP

These observed metabolic changes taught us that PHD1 deficiency has diverging cell-type specific effects on cellular metabolism. However, they did not provide an immediate explanation as to why PHD1$^{-/-}$ neurons are protected against ischemia. Previous work pointed out that glycolytic flux in neurons is kept at a low rate to enable a sufficient flux of glucose through the oxidative pentose phosphate pathway (oxPPP) (see also chapter I) (Herrero-Mendez et al 2009). The PPP is an alternative route for glucose-6-phosphate besides glycolysis. The oxPPP refers to the oxidative branch of this pathway where glucose oxidation converts [$NADP^+$] to [NADPH]. NADPH produced by the oxPPP was recently shown to be of vital importance in replenishing the pool of reduced glutathione (GSH) and maintaining redox homeostasis in neurons (Herrero-Mendez et al 2009).

Given this reciprocal regulation of glycolysis and the oxPPP, we hypothesized that the reduced glycolytic flux was part of glucose rerouting towards the oxPPP. To investigate whether PHD1 affects the oxPPP, we measured $^{14}CO_2$ production upon incubation with [6-^{14}C]-glucose (producing $^{14}CO_2$ only in the TCA cycle) or [1-^{14}C]-glucose (producing $^{14}CO_2$ in both the TCA cycle and PPP) and subtracted the [6-^{14}C]-glucose flux from the [1-^{14}C]-glucose flux. This analysis revealed a more than 2-fold induction in the absolute flux levels of the oxPPP in PHD1$^{-/-}$ neurons (pmol glucose/h/10^6 cells: 190 ± 44 for wild type *versus* 467 ± 51 for PHD1$^{-/-}$ neurons; N=4; P<0.01; Figure 12A,B)†. When calculating the relative flux by correcting for the level of the glycolytic flux (reflecting the contribution of the oxPPP to glucose metabolism), the relative oxPPP flux was even increased 8-fold (Figure 12C). These findings suggested that PHD1 deficiency shifts glucose from glycolysis towards the oxPPP.

† Note that the absolute PPP flux levels in (B) are lower than generally reported in the literature. This is probably due to the usage of an open-air system (increasing the escape of $^{14}CO_2$ during the incubation).

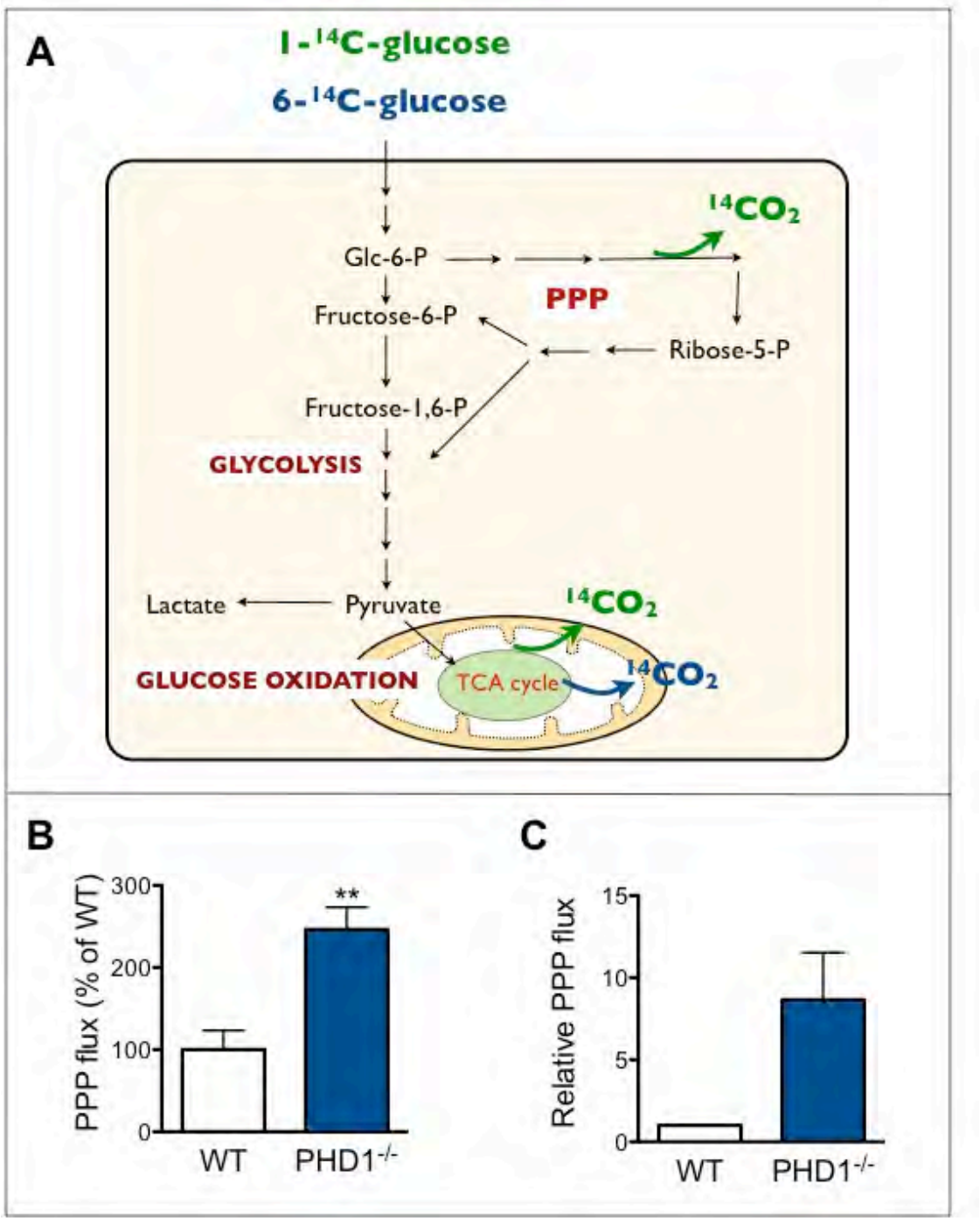

Figure 12: Enhanced oxPPP flux in the PHD1$^{-/-}$ neurons.
(A) Schematic overview of oxPPP-flux measurements. Incubation with 1-^{14}C-glucose (in green) results in $^{14}CO_2$ formation in the oxPPP but also in the TCA cycle whereas 6-^{14}C-glucose (blue) incubation will result in $^{14}CO_2$ release only in the TCA. Therefore, the 6-^{14}C-glucose flux subtracted from the 1-^{14}C-glucose flux reflects the flux through the oxPPP. (B) Relative (%) change of absolute oxPPP flux in PHD1$^{-/-}$ neurons compared to WT neurons (N=4; **P<0.01). (C) Relative oxPPP flux in WT and PHD1$^{-/-}$ neurons (N=4).

PHD1 DEFICIENCY IMPROVES ROS SCAVENGING CAPACITY IN NEURONS

Glutathione peroxidase detoxifies H_2O_2 to H_2O by converting reduced glutathione (GSH) into its oxidized form (GSSG). GSH is then regenerated through oxidation of [NADPH] by glutathione reductase. The increased oxPPP flux would thus imply an improved scavenging capacity of H_2O_2 and oxygen radicals in general. To explore this possibility, we exposed neurons, labeled with CM-H_2DCF (a dye measuring ROS levels), to 100 µM H_2O_2 and measured H_2O_2 levels over time. In this way, the detoxification rate of H_2O_2 and hence the recycling rate of glutathione reduction can be assessed. Compared to WT neurons, PHD1$^{-/-}$ neurons showed a less steep increase in CM-H_2DCF fluorescence intensity over time (Figure 13A), implying accelerated detoxification of the administered H_2O_2 molecules.

Next, we investigated whether the enhanced oxPPP flux, via accelerating the glutathione recycling, was responsible for the improved ROS scavenging capacity. Therefore, we blocked the oxPPP flux by silencing glucose-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the oxPPP flux (Figure 13B). Using a lentiviral shRNA against G6PD, a knockdown efficiency of 80% was reached, and the oxPPP flux in PHD1$^{-/-}$ neurons was nullified (Figure 13C). G6PD silencing deteriorated the scavenging capacity of PHD1$^{-/-}$ neurons (Figure 13D), suggesting that their improved ROS scavenging capacity is, at least partially, dependent on their increase in oxPPP flux. As G6PD silenced PHD1$^{-/-}$ neurons maintained a tendency of a slight improved scavenging potential, it is possible that PHD1 deficiency enables other (compensatory) mechanisms allowing ROS scavenging upon PPP blockade. Gene expression analysis of various anti-oxidant enzymes did not show enhanced expression of the most prevalent anti-oxidant-enzymes in neurons (table 3).

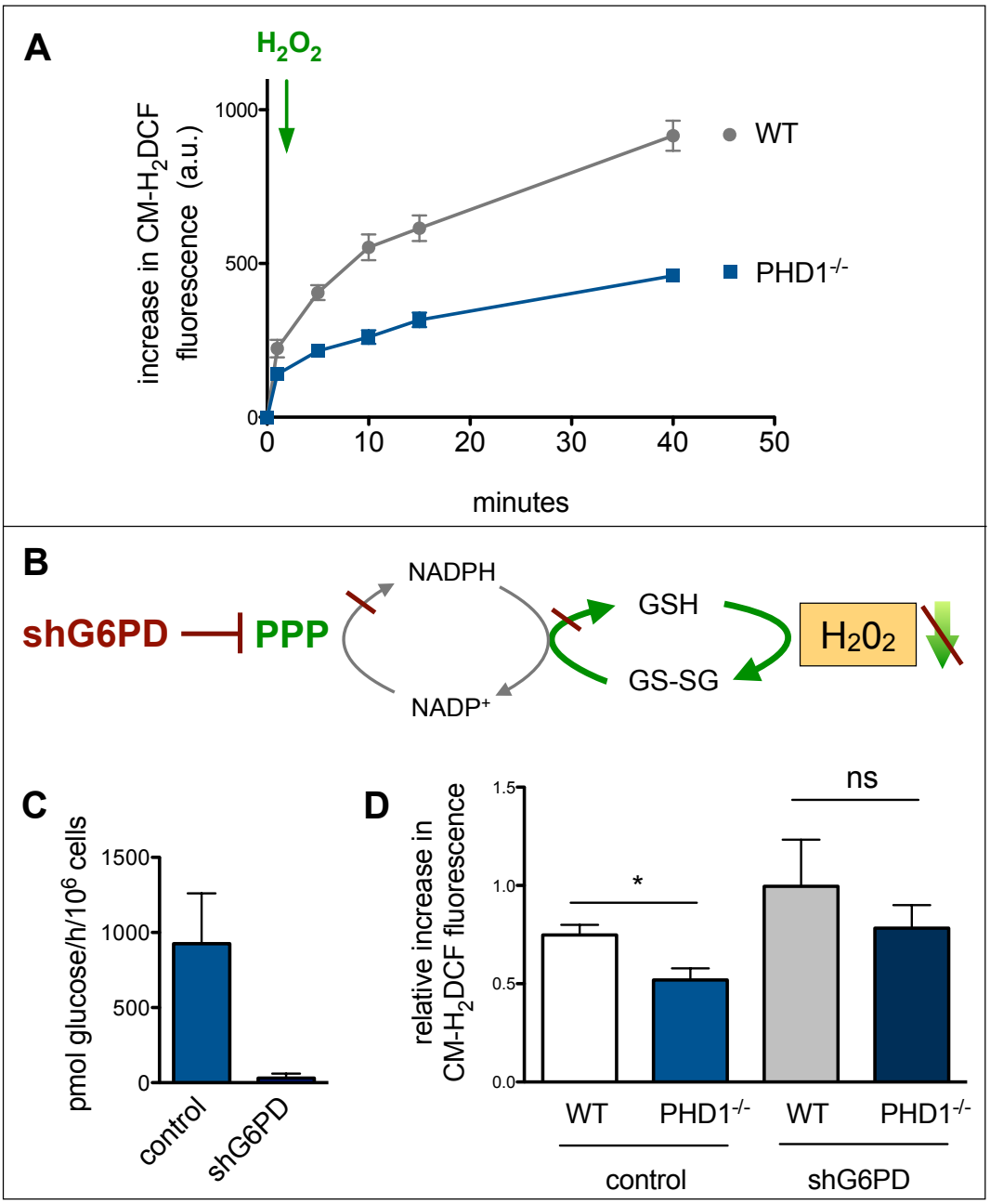

Figure 13: PHD1 deficiency and oxPPP flux improve the scavenging capacity of oxygen radicals in neurons.
(A) Representative time course of the increase in CM-H_2DCF fluorescence depicting ROS levels over time after administration of H_2O_2 in WT and PHD1$^{-/-}$ neurons (N=6; ****P<0.0001, two-way ANOVA). a.u.: arbitrary units. (B) Scheme showing consequences of silencing G6PD (shG6PD): inhibition of PPP flux leads to a lower glutathione reduction rate and hence less detoxification of H_2O_2. (C) oxPPP flux in control and G6PD silenced PHD1$^{-/-}$ neurons (N=3; P=0.056). (D) Relative increase in CM-H_2DCF fluorescence 20 minutes after H_2O_2 administration to WT and PHD1$^{-/-}$ neurons (N=3; *P<0.05 for WT *versus* PHD1$^{-/-}$ neurons; P=0.47 for G6PD silenced WT and PHD1$^{-/-}$ neurons).

Table 3: Gene expression analysis of anti-oxidant enzymes

GENE	WT NEURONS	PHD1$^{-/-}$ NEURONS
GCLC	1.00 ± 0.10	0.84 ± 0.04
Gpx1	1.00 ± 0.04	0.98 ± 0.09
Gpx4	1.00 ± 0.04	1.08 ± 0.02
Prdx1	1.00 ± 0.05	0.99 ± 0.10
Prdx3	1.00 ± 0.14	1.21 ± 0.25
Prdx4	1.00 ± 0.09	1.26 ± 0.16
Sesn1	1.00 ± 0.02	0.94 ± 0.08
Sesn2	1.00 ± 0.11	1.26 ± 0.04
SOD1	1.00 ± 0.07	0.93 ± 0.14
SOD2	1.00 ± 0.10	0.86 ± 0.11

Quantitative RT-PCR data of different anti-oxidant genes in WT and PHD1$^{-/-}$ neurons in baseline conditions. Representative experiments of fold expression in the PHD1$^{-/-}$ neurons compared to wild type (WT) neurons are shown (mean± SEM, N=3-6, P=ns). GCLC: glutamate-cystein ligase catalytic subunit; Gpx: glutathione peroxidase; Prdx: peroxiredoxin; Sesn: sestrin; SOD: superoxide dismutase.

PHD1 DEFICIENCY COUNTERACTS HYPOXIC METABOLISM IN NEURONS

In various cell types, it is firmly established that hypoxia induces a HIF-1α mediated increase in glycolytic flux (see chapter I). Also in cultured neurons, we observed an elevated glycolytic flux upon hypoxic exposure. In line with this, HIF-1 protein levels and mRNA levels of various glycolytic genes were upregulated under these circumstances (data not shown). Even though PHD1$^{-/-}$ neurons also responded to hypoxia by increasing their glycolytic flux, the glycolytic flux rate remained lower in hypoxic PHD1$^{-/-}$ than in WT neurons

(Figure 14A).

Given the reciprocal regulation of glycolysis and oxPPP, we questioned how hypoxia would affect the oxPPP flux. Strikingly, hypoxia nullified the oxPPP flux in wild type neurons. PHD1$^{-/-}$ neurons however were able to maintain a measurable oxPPP flux, although it was smaller than in normoxic conditions (Figure 14B). These data suggest that PHD1 deficiency attenuates the hypoxia-induced metabolic shift in which glycolysis increases at the expense of the oxPPP flux. By reprogramming glucose metabolism and maintaining a oxPPP flux, PHD1 deficiency ensures redox homeostasis, especially during ischemic challenges.

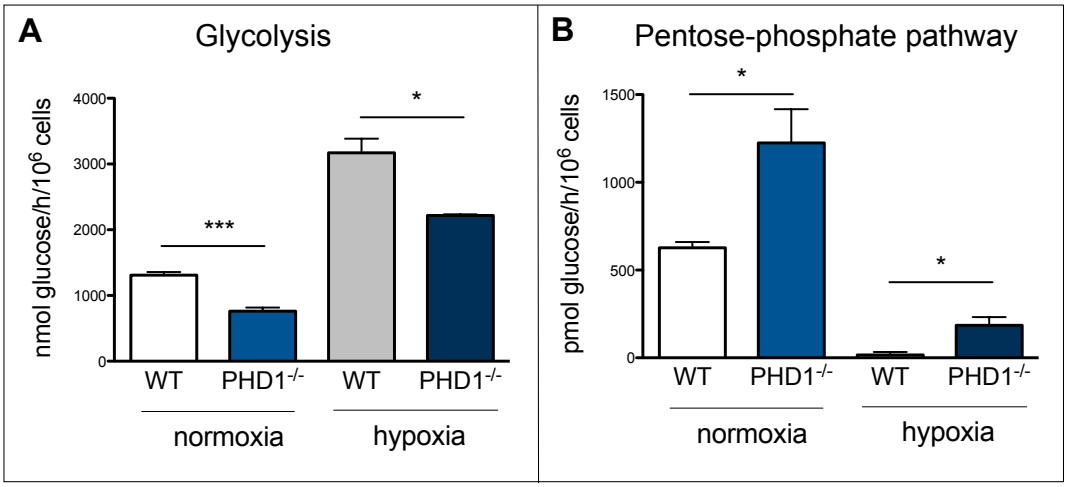

Figure 14: PHD1 deficiency attenuates hypoxia-induced metabolic changes in neurons
(A) Representative experiment of relative (%) change of glycolytic flux in PHD1$^{-/-}$ neurons compared to WT neurons in normoxic and hypoxic conditions (0.1% O_2 for 2 hours) (N=4; *P<0.05, **P<0.01 and ***P<0.001). (B) Relative (%) change of oxidative PPP flux in PHD1$^{-/-}$ neurons compared to WT neurons in normoxic and hypoxic conditions (0.1% O_2 for 2 hours) (N=3, *P<0.05).

PHD1$^{-/-}$ NEURONS OXIDIZE MORE GLUTAMINE

So far, we described that a reduction of glycolysis was linked to an increase in PPP and to a decreased oxidation of glucose in the mitochondria. At first sight, it might be expected that a reduction in glucose oxidation would threaten the energy homeostasis and jeopardize neuronal survival. However, given the comparable oxygen consumption rates in wild type and PHD1$^{-/-}$ neurons, we hypothesized that as compensation, oxidation of alternative substrates would be enhanced. Previous studies indeed established that neurons oxidize not only glucose, but also lactate and glutamine, provided by their neighboring cells (Mergenthaler et al 2013).

As mentioned earlier, the oxidation of lactate was unaffected in PHD1$^{-/-}$ neurons. Glutamine is another energy substrate that after conversion first to glutamate by glutaminases (GLS1 and GLS2) and, thereafter, to α-ketoglutarate (αKG) by glutamate dehydrogenase (Glud), will enter the TCA cycle. Here it is subjected to different oxidation steps that produce CO_2 and generate energy producing NADH molecules. To determine whether glutamine was oxidized differently in PHD1$^{-/-}$ neurons, we incubated cells with [U-^{14}C] glutamine and monitored the release of $^{14}CO_2$. These measurements revealed that glutamine oxidation rates were modestly enhanced in PHD1$^{-/-}$ neurons (pmol glutamine/h/10^6 cells: 1,144 ± 73 for WT *versus* 1,564 ± 88 for PHD1$^{-/-}$ neurons; N=3; *P<0.05). Gene expression analysis of enzymes involved in glutamine metabolism showed a modest increase in *Gls2* mRNA levels in PHD1$^{-/-}$ neurons (relative mRNA expression: 1.00 ± 0.05 in WT neurons *versus* 1.57 ± 0.14 for PHD1$^{-/-}$ neurons; N=3-4; **P<0.01) (table 4).

Taken together, these data suggest that the decrease in ATP production due to the reduction of glucose oxidation was compensated by an enhanced production of ATP resulting from increased glutamine oxidation. Indeed, PHD1 deficiency did not alter the energy charge balance ([ATP] + ½ [ADP]) / ([ATP] + [ADP] + [AMP]) (0.95 ± 0.001 for WT *versus* 0.95 ± 0.001 for PHD1$^{-/-}$ neurons; N=4; P=0.72), indicating that PHD1$^{-/-}$ neurons were not in

energy distress.

Table 4: Gene expression analysis of glutamine metabolism

GENE	WT NEURONS	PHD1$^{-/-}$ NEURONS
Gls1	1.00 ± 0.11	1.01 ± 0.05
Gls2	1.00 ± 0.05	1.57 ± 0.14 **
Glud	1.00 ± 0.06	0.92 ± 0.08

Quantitative RT-PCR analysis (representative experiment) of the expression of different genes involved in glutamine metabolism in WT and PHD1$^{-/-}$ neurons (baseline conditions). Data are expressed relative to WT neurons (mean±SEM, N=3-4, **P<0.01). Gls: glutaminase; Glud: glutamate dehydrogenase.

GLYCOGEN AS A POSSIBLE SOURCE OF ENERGY IN PHD1$^{-/-}$ NEURONS DURING ISCHEMIA

Our observations so far demonstrated that loss of PHD1 enhanced neuronal anti-oxidant defense mechanisms. We next questioned whether besides enhanced ROS scavenging capacity (which would protect neurons especially during the reperfusion period), PHD1$^{-/-}$ neurons also possess alternative mechanisms to counter ATP depletion during oxygen-nutrient deprivation. To address this possibility, we deprived WT and PHD1$^{-/-}$ neurons for 8 hours from oxygen (0.1% O_2) and nutrients (glucose, glutamine and pyruvate) and monitored their energy charge. While in WT neurons the energy charge declined more than 50% after 8 hours, indicating severe energetic stress, loss of PHD1 enabled a better maintenance of the energy charge during oxygen-nutrient deprivation (Figure 15). Thus, PHD1$^{-/-}$ neurons are more resistant to prolonged starvation.

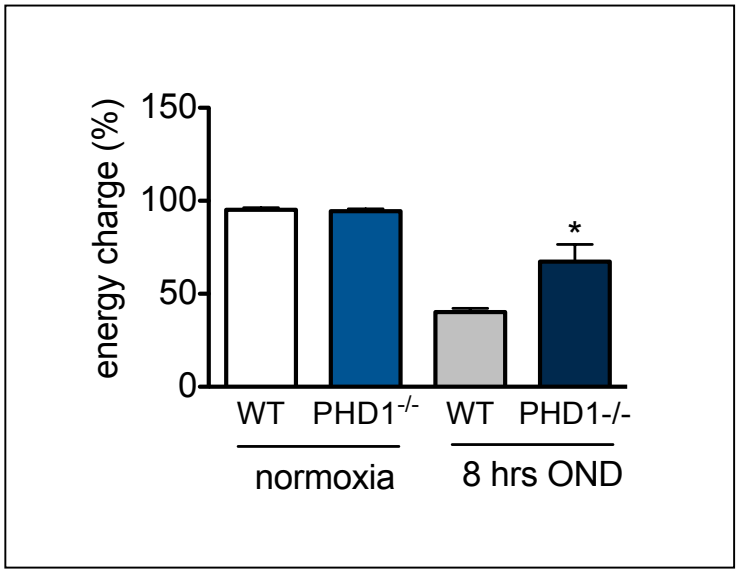

Figure 15: PHD1 deficiency improves energy charge maintenance during prolonged oxygen-nutrient deprivation
Energy charge measured in WT and PHD1$^{-/-}$ neurons in normoxic conditions and after 8 hours of oxygen-nutrient deprivation (OND) (for normoxia: N=3; P=0.7; for 8 hrs OND: N=3; *P<0.05).

These data strongly suggests that PHD1$^{-/-}$ neurons possess an alternative energy store that can be recruited whenever extracellular nutrients are lacking. Increased autophagy would theoretically be an alternative protective mechanism in nutrient starvation. However, numerous papers have indicated that in cultured neurons the activation of the autophagy pathway is contributive to neuronal cell death rather than being protective (Degterev et al 2005, Shi et al 2012, Wen et al 2008).

The most plausible internal energy store would be glycogen, even though it has been debated whether or not neurons could store glycogen (DiNuzzo et al 2011). A recent report showed that neurons do accumulate limited stores of glycogen, which can be utilized during ischemia (Saez et al 2014). We used the Periodic Acid Schaff (PAS) staining to assess the glycogen content. Initial data show that WT neurons indeed contained few and faint PAS-positive granules in their cytoplasm (Figure 16A-C), whereas PHD1$^{-/-}$ neurons showed more PAS-positive granules with higher staining intensity (Figure 16D-F).

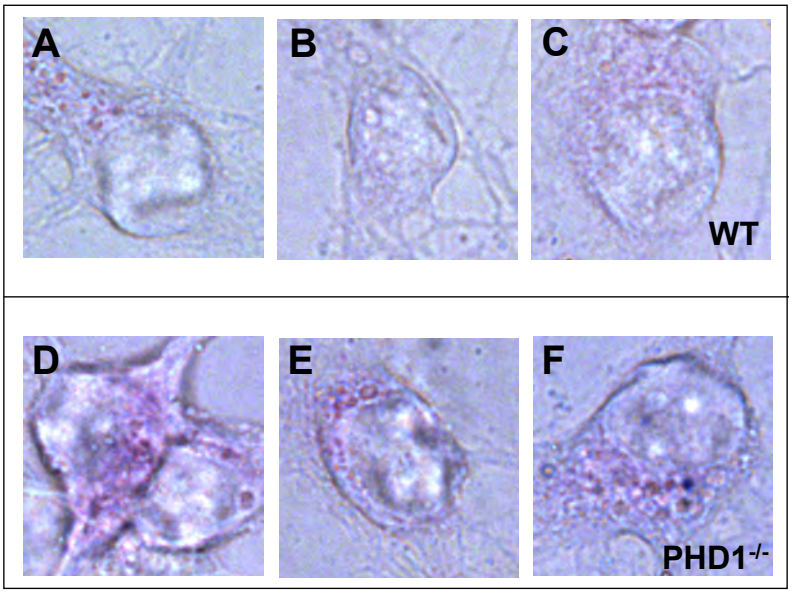

Figure 16: PHD1$^{-/-}$ neurons have a higher glycogen content.
Representative images of a PAS-staining in WT and PHD1$^{-/-}$ neurons. (A-C) WT neurons containing some faint PAS-positive granules in their cytoplasm. (D-F) PHD1$^{-/-}$ neurons showing more and larger PAS-positive granules with higher staining intensity (Figure 17D-F).

ISCHEMIC PROTECTION IN PHD1$^{-/-}$ NEURONS IS HIF-INDEPENDENT

HIF-1α and HIF-2α are the two best-characterized hydroxylation targets of PHDs. In different organs and tissues, HIF-1α and/or HIF-2α were shown to mediate the effects of PHD deficiency, even though HIF-independent effects have been increasingly recognized (Quaegebeur and Carmeliet 2010, Wong et al 2011). In normoxic PHD1$^{-/-}$ neurons, given the lack of hydroxylation activity by PHD1, one would expect increased levels of HIF-1α and HIF-2α. Immunoblotting showed that levels of HIF-1α were not elevated in normoxic PHD1$^{-/-}$ neurons nor in PHD1$^{-/-}$ brain (Figure 17A,B). In repeated attempts, HIF-2α protein could not be visualized in normoxic conditions.

Additionally, we explored the potential involvement of HIF-1α and HIF-2α by assessing the effect of HIF-1α and HIF-2α silencing on PHD1 deficient neuronal protection. Silencing of HIF-1α via a lentiviral vector expressing a shRNA against HIF-1α did not abolish the protection against oxygen-nutrient deprivation by PHD1 deficiency, nor did a shRNA against HIF-2α (Figure 17C). Interestingly, lowering HIF-1α levels in *in vitro* ischemia even attenuated neuronal cell death both in wild type and PHD1$^{-/-}$ neurons. Taken together, these data point towards a HIF-independent mechanism.

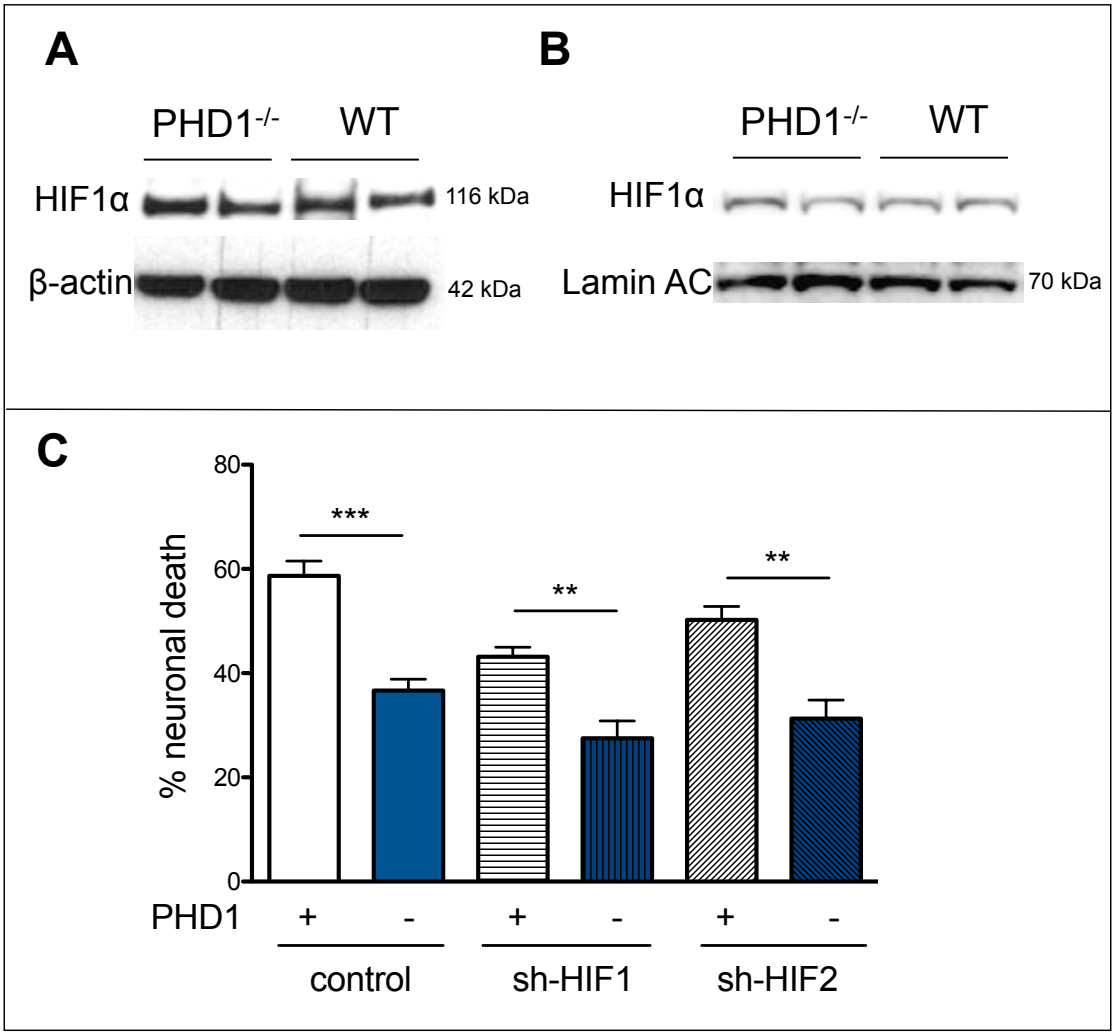

Figure 17: HIF-1α levels are not induced in PHD1$^{-/-}$ neurons and neither HIF-1α or HIF-2α explain the ischemic protection

(A-B) Representative western blots showing protein levels of HIF-1α in WT and PHD1$^{-/-}$ normoxic cortical neurons (A) and normoxic brains (B). (C) Representative experiment showing neuronal cell death measured as LDH release (% of maximal LDH release) over 24 hours after oxygen-nutrient deprivation in WT and PHD1$^{-/-}$ neurons upon HIF-1α and HIF-2α silencing (N=5-6, **P<0.01, ***P<0.001).

INTRACEREBROVENTRICULAR DELIVERY OF ANTI-PHD1-DIRECTED OLIGONUCLEOTIDES PROTECTS AGAINST BRAIN ISCHEMIA

We intended to explore the translation potential of PHD1 inhibition in ischemic stroke. PHD1 inhibition might be an attractive target for different reasons. First of all, genetic deletion of PHD1 results in a pronounced protection against brain ischemia. Additionally and of paramount importance, PHD1 loss does not result in major deficits in mice, as was shown in previous studies (Aragones et al 2008, Schneider et al 2009, Tambuwala et al 2010). This suggests that PHD1-specific inhibitors might have a more favorable side-effect profile than the available aspecific PHD inhibitors. Since at the initiation of this study no specific PHD1 inhibitor was available, we turned to the possibility of delivering antisense oligonucleotides (ASOs). The oligonucleotides were designed and generated by Isis Pharmaceuticals as previously described (Bennett and Swayze 2010). A library of ASOs was screened for targeting murine PHD1 and three different ASOs were selected for further research (by Isis Pharmaceuticals). Out of these three, we selected the ASO with the highest knockdown efficiency and specificity to use in our *in vivo* studies.

First, we tested the selected ASO *in vitro*. After incubation of cultured cortical neurons with 0.5 μM[**] of the anti-PHD1 ASO for 5 days, a knockdown efficiency of 85% was obtained. We additionally determined PHD2 and PHD3 mRNA levels in these cultures, which remained unaffected (Figure 18A). This confirmed the efficacy and specificity for PHD1 of this ASO.

In a next step, we investigated the feasibility of *in vivo* delivery of ASOs. Since the blood-brain barrier is not permeable for ASOs (Broaddus et al 2000, Cossum et al 1993, Smith et al 2006), we chose intracerebroventricular delivery via osmotic pumps as previously described (Storkebaum et al 2005). The ASOs were continuously infused over the course of 2 weeks at a rate of

[**] This concentration was chosen based on advice from Isis Pharmaceuticals and after a dose-response experiment identifying 0.5 μM as the concentration with the highest PHD1 knockdown efficiency and specificity.

75 µg/day, a dose which was based on the advice of Isis Pharmaceuticals. The animals tolerated this treatment well and no premature deaths were noted. After these 2 weeks, brains were harvested and PHD1 mRNA levels were assessed. This analysis showed that intracerebroventricular delivery of anti-PHD1 ASOs lowered PHD1 brain mRNA levels to 50% of control treated animals (Figure 18B).

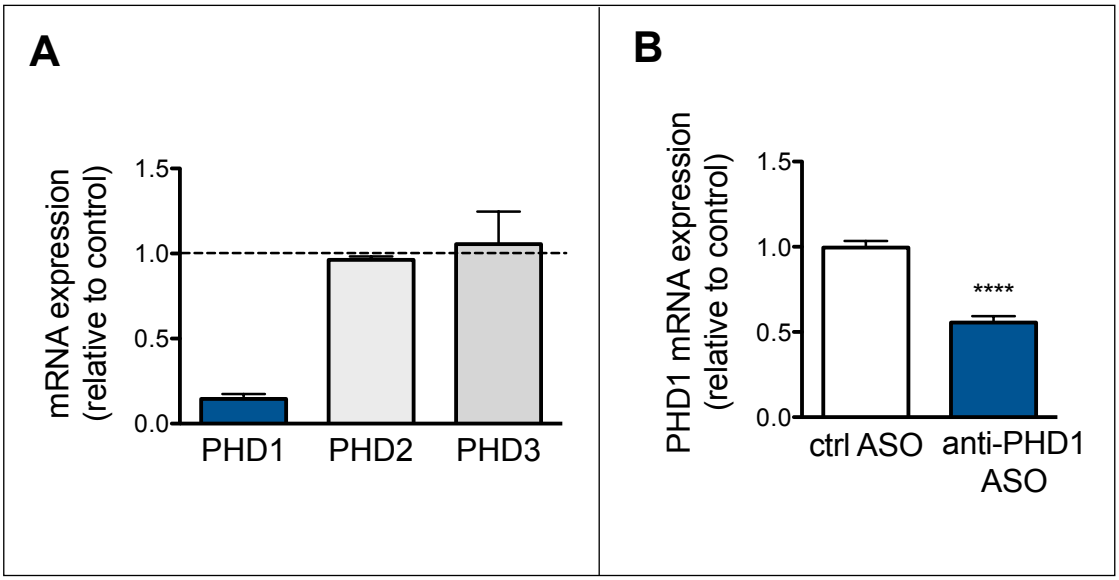

Figure 18: Anti-PHD1 oligonucleotides lower PHD1 levels in cultured cortical neurons and in brain tissue
(A) mRNA expression levels of PHD1, PHD2 and PHD3 in neuronal cultures incubated with anti-PHD1 oligonucleotides (0.5 µM) for 5 days, relative to levels in neuronal cultures incubated with control oligonucleotides (0.5 µM). (B) mRNA expression levels of PHD1 in brain homogenates from control treated mice and anti-PHD1 oligonucleotide treated mice (N=4-5; ****P<0.0001).

Given the successful knockdown of PHD1 in the brain, we tested the effect of ASO delivery on brain ischemia. Mice treated for 2 weeks with anti-PHD1 ASOs underwent a permanent ligation of their middle cerebral artery and infarct size was assessed 24 hours later. Mice with intracerebroventricular delivery of saline or of control ASOs were used as two control groups. This

control ASO was directed against human huntingtin mRNA and was chemically modified to prevent any RNase H-mediated effects (personal communication with Isis Pharmaceuticals). Infarct size analysis showed a 50% reduction in stroke lesion upon treatment with the anti-PHD1 ASOs (Figure 19A-J). Of importance, no difference in stroke size was observed between the saline and control ASO group, demonstrating that injection of an oligonucleotide per se did not cause additional effects that would influence stroke outcome.

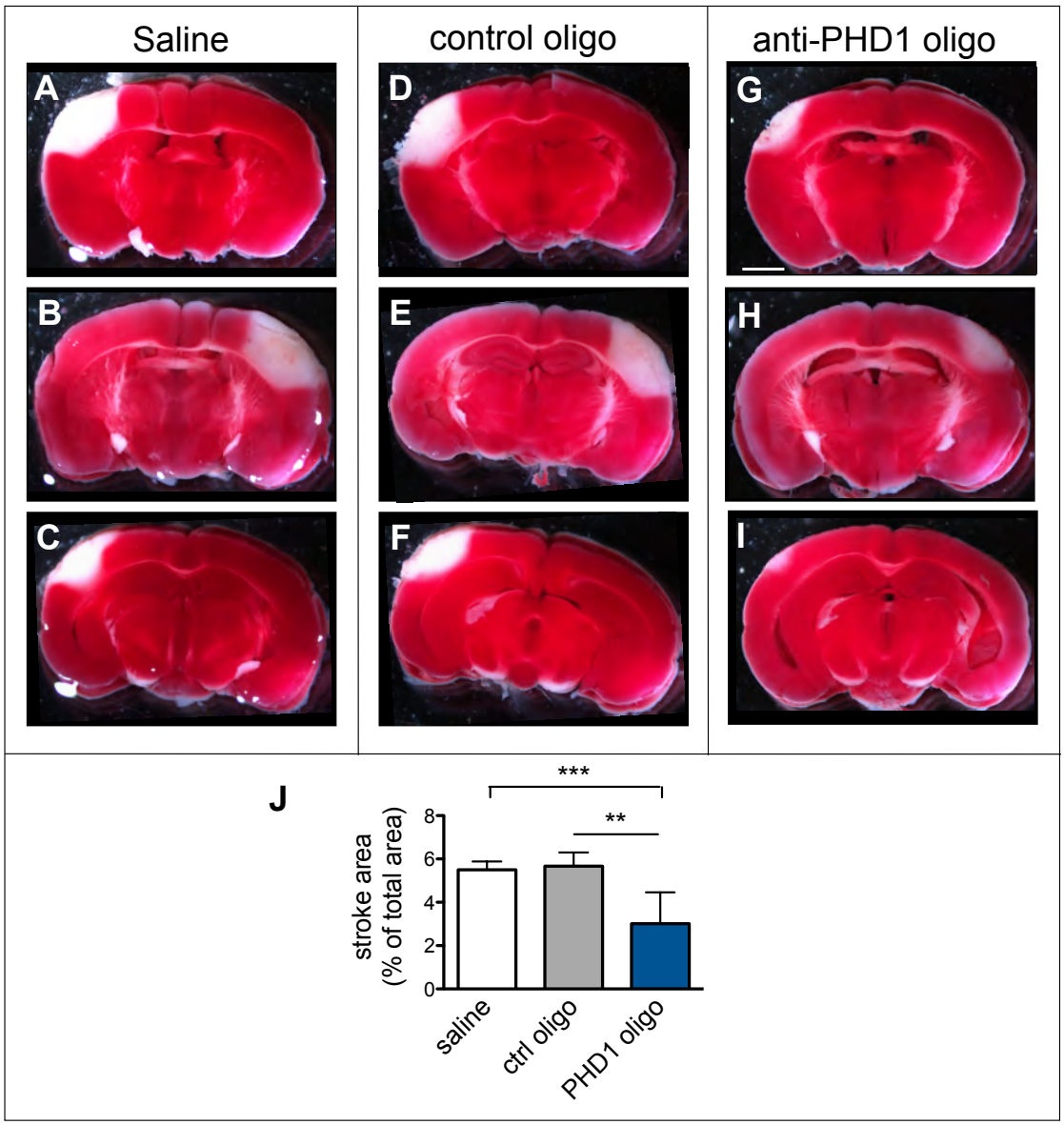

Figure 19: PHD1 knockdown by anti-PHD1 oligonucleotides reduces the infarct size after pMCAO
Representative brain slices after TTC staining, delineating the infarct zone as unstained area (white), from control mice receiving saline (A-C), control mice receiving human control oligonucleotides (D-F) and mice receiving anti-PHD1 directed oligonucleotides (G-I), 24 hours after pMCAO. Quantification of the stroke area as percentage of the total bihemispheric area is shown in J (N=8 for the saline group, N=5 for the control oligo group, N=9 for the anti-PHD1 oligo group, $^{**}p<0.01$ for control oligo *versus* anti-PHD1 oligo and $^{***}p<0.001$ for saline *versus* anti-PHD1 oligo). Scale bar: 1 mm.

Future research will have to point out whether PHD1 inhibition would also be a valid neuroprotective target when initiated after the onset of the stroke. The development and characterization of selective (preferably BBB-permeable) PHD1 inhibitors will be crucial in shaping clinical indications for PHD1 inhibition and directing further studies.

PHD1 DEFICIENCY ATTENUATES NEURODEGENERATION IN A MOUSE MODEL OF ALS

Energy depletion and oxidative stress are common players not only in acute ischemic stroke but also in neurodegenerative conditions with a more chronic disease course. We therefore hypothesized that PHD1 deficiency might also be beneficial in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and Huntington's disease. We decided to focus our initial exploration on ALS (a neurodegenerative disease characterized by predominant degeneration of motorneurons). Next to Alzheimer's disease, ALS is a neurodegenerative disorder with the strongest evidence that vascular deficits (e.g. impaired angiogenic responses, disregulation of vasomotor responses, BBB leakiness, …) and subsequent hypoxic events are majorly implicated in disease pathogenesis (Quaegebeur and Carmeliet 2010). We intercrossed $PHD1^{-/-}$ mice with $SOD1^{G93A}$ mice, a disease model for ALS based on overexpression of a human mutation in SOD1. This mutation is believed to result in degeneration of motorneurons via a gain-of-function mechanism. We compared paralysis onset (as defined by failed performance on the rotarod) and survival between $PHD1^{-/-}$ $SOD1^{G93A}$ and $PHD1^{+/+}$ $SOD1^{G93A}$ littermates (females only). $PHD1^{+/+}$ $SOD1^{G93A}$ mice lived on average 132 days, whereas $PHD1^{-/-}$ $SOD1^{G93A}$ mice were found to have an extended life span of, on average, 15 days ($PHD1^{+/+}$ $SOD1^{G93A}$ mice: 132±2 days, N=16; $PHD1^{-/-}$ $SOD1^{G93A}$ mice: 147±6 days, N=11; $P<0.01$; Figure 20A). PHD1 deficiency also resulted in a delay in paralysis onset, even though this effect was less pronounced than the effect on survival ($PHD1^{+/+}$ $SOD1^{G93A}$ mice: 123±2 days, n=15; $PHD1^{-/-}$ $SOD1^{G93A}$ mice: 132±5 days, N=11; $P<0.05$; Figure 20B).

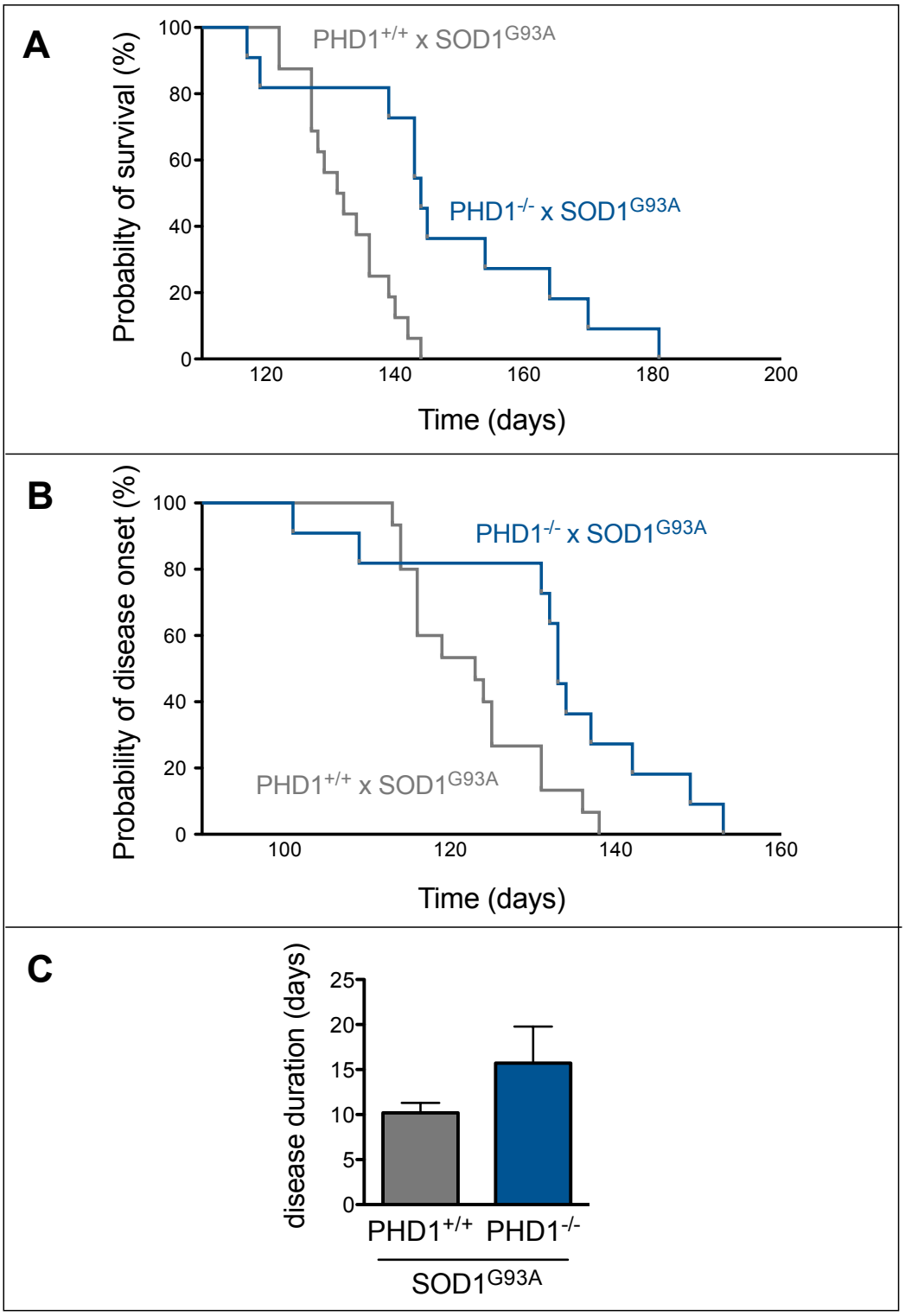

Figure 20: PHD1 deficiency has a beneficial effect on survival and motor performance of SOD1^{G93A} mice

(A) Kaplan-Meier curve showing probability of survival over time in PHD1$^{-/-}$ SOD1^{G93A} (blue) and PHD1$^{+/+}$ SOD1^{G93A} littermates (grey). Median survival is 144 days for PHD1$^{-/-}$ SOD1^{G93A} mice (N=11) and 131.5 days for PHD1$^{+/+}$ SOD1^{G93A} mice (N=16) (Log-Rank test P<0.001). (B) Kaplan-Meier curve

showing probability of paralysis onset (defined as rotarod failure) over time in $PHD1^{-/-}$ $SOD1^{G93A}$ (blue) and $PHD1^{+/+}$ $SOD1^{G93A}$ littermates (grey). Median paralysis onset is 133 days for $PHD1^{-/-}$ $SOD1^{G93A}$ (N=11) and 123 days for $PHD1^{+/+}$ $SOD1^{G93A}$ (N=15) (Log-Rank test $P<0.05$). (C) Disease duration (defined as the interval between rotarod failure and survival) in $PHD1^{+/+}$ $SOD1^{G93A}$ (grey) and $PHD1^{-/-}$ $SOD1^{G93A}$ (blue) littermates (N=11 for $PHD1^{-/-}$ $SOD1^{G93A}$ and N =15 $PHD1^{+/+}$ $SOD1^{G93A}$ littermates; P=0.12)

The larger effect on survival than on disease onset also implicates that PHD1 deficiency attenuated disease progression, which is of importance from a therapeutic perspective: $PHD1^{-/-}$ $SOD1^{G93A}$ mice showed a trend of a 60% increase in their disease duration ($PHD1^{+/+}$ $SOD1^{G93A}$ mice: 10±1 days, N=14; $PHD1^{-/-}$ $SOD1^{G93A}$ mice: 16±2 days, N=11; P=0.12; Figure 21C).

Our observation that genetic deletion of PHD1 has a beneficial effect on the disease course in an ALS mouse model might be a paradigm for PHD1 inhibition in neurodegenerative disorders. Whether metabolic changes in motorneurons underlie the protective effect, whether anti-PHD1 oligonucleotides can phenocopy the beneficial effect of genetic deletion, and whether PHD1 inhibition would be a valuable therapy for other neurodegenerative disorders, remain outstanding questions.

Chapter V

DISCUSSION

The primary finding of this study is that deletion of the oxygen sensor PHD1 largely prevents brain ischemia following ligation of the middle cerebral artery in mice. This was not due to vascular alterations in the PHD1 deficient brain; instead neurons lacking PHD1 rewire their metabolism, which enables them to tolerate energy depletion and oxidative stress. In addition, we show that acute inhibition of PHD1 by oligonucleotide-mediated silencing mimics the protective effect of genetic deletion in ischemic stroke. This is - to the best of my knowledge - the first preclinical stroke study in which the effect of acute pharmacological inhibition of a single PHD isoform was investigated.

1 Genetic deletion of PHD1 in brain and neurons protects against acute ischemic stroke

There is ample genetic and pharmacological evidence for a protective role of PHD inhibition in ischemic disorders. However, the current PHD inhibitors have an unfavorable side effect profile due to their lack in specificity. Therefore, there is a compelling need for a more profound understanding of the isoform-specific roles and underlying molecular mechanisms in different organs before PHD inhibition will be translatable into a valid and clinically applicable therapeutic strategy.

In this study, we reported that genetic deletion of PHD1 is protective against brain ischemia. This protective effect was reflected in a 70% smaller infarct size 24 hours after permanent ligation of the middle cerebral artery and was observed in two different mouse strains. Of importance, loss of PHD1

nearly completely prevented the functional sensorimotor deficits after stroke, an observation that remained over a prolonged period of time. The stroke size was not affected by PHD2 or PHD3 deficiency, indicating that, in the condition of permanent brain ischemia, PHD1 is the main oxygen sensor. Two recent studies reported however a beneficial effect of PHD2 deficiency in stroke, albeit in another, transient, model of cerebral ischemia. Neuron-specific PHD2 knockout mice (by using CAMKII cre mice) were protected against cell death after transient cerebral ischemia (Kunze et al 2012) and heterozygous deficient PHD2 mice showed a trend of reduced infarct size after transient middle cerebral artery occlusion (tMCAO) (Chen et al 2012).

Several reasons could possibly reconcile these reports with our observations that mice with a neural-specific deletion of PHD2 (by using Nestin Cre $PHD2^{lox/lox}$ mice, deleting PHD2 in neurons but also in glia) are not protected against stroke. First of all, transient occlusion with subsequent reperfusion is believed to involve other cellular and biochemical events preceding neuronal death than permanent ischemia. This jeopardizes the comparison of the effects in these two models (Hossmann 2012). Also, imaging, molecular and biochemical studies showed differences in the temporal evolution of penumbral and necrotic tissue in transient MCAO *versus* permanent MCAO. After permanent ligation of the middle cerebral artery, the final ischemic lesion can be determined 24 hours later (Hata et al 2000a), whereas the evolution after transient occlusion is more protracted with the ischemic lesion being final only after 3 days (Hata et al 2000b). In this regard, one could criticize the 24-hour time point used in the Kunze and the Chen study to assess infarct size (Chen et al 2012, Kunze et al 2012), since at this point the evolution of the ischemic lesion would still be incomplete (Hata et al 2000b).

Secondly, all three studies used different approaches to genetically silence PHD2: whereas neuron-specific deletion of PHD2 had a substantial effect on brain ischemia (Kunze et al 2012), heterozygous deficiency caused only a trend of reduced infarct size (Chen et al 2012), while neural-specific

PHD2 deletion in our study did not yield any difference. This underscores once more the cell-type specific (Nestin Cre *versus* CamKII cre) and dose-dependent effects of PHDs. Finally, the study showing a partial protection by heterozygous PHD2 deficiency also reported a similar trend in PHD1$^{-/-}$ mice (Chen et al 2012). An important remark here is that this study used PHD1$^{-/-}$ mice in a mixed Swiss/129S6 background as compared to a pure 129S6 and C57/Bl6 background in our study, which might have modified the results. Since a strain-specific vulnerability is widely recognized in the field of experimental stroke, the usage of pure background strains is essential (Fujii et al 1997). From these studies it is unclear which mechanism is explanatory for the protective effect of PHD2 deficiency. In one study, PHD2 deficient mice showed a more rapid restoration of blood flow after reperfusion.

2 Is loss of PHD1 neuroprotective via extrinsic (vascular) mechanisms or by modulating intrinsic (neuronal) features?

By promoting blood supply or by modulating the intrinsic resilience of cells against hypoxia and oxidative stress, PHDs are the master regulators of the adaptive response to hypoxic stress. Stroke outcome has extensively been linked to the extent of residual perfusion, which is determined by the degree of collateral circulation (Shuaib et al 2011). Upon acute occlusion of the middle cerebral artery, pial collateral vessels can, via retrograde filling, still supply brain regions distal to the occlusion, alleviating the drop in perfusion (Brozici et al 2003). Studies in both experimental models (Chalothorn and Faber 2010, Defazio et al 2011, Zhang et al 2010) and patients (Bang et al 2008, Kucinski et al 2003, Lima et al 2010, Maas et al 2009, Miteff et al 2009, Shuaib et al 2011) pinpointed the collateral circulation as an important determinant of stroke size and outcome.

PHDs have been previously linked to arteriogenic remodeling and improved collateral flow (Takeda et al 2011). Also, chronic cerebral hypoxia was recently shown to promote arteriogenesis in the brain (Boroujerdi et al

2012, Freitas-Andrade et al 2012). We did not observe any effect of PHD1 deficiency on the number of pial collaterals (responsible for the residual perfusion in experimental MCAO models (Zhang et al 2010)). Also, neither vessel perfusion nor arterioles were affected in the PHD1$^{-/-}$ brain. All these vascular parameters were assessed in non-stroked animals, since a possible contribution of vascular preconditioning would imply them to be altered prior to the occlusion. The lack of a clear vascular phenotype in the PHD1$^{-/-}$ brains is compatible with the findings in other PHD1 deficient organs, where vascular effects have never been described either (Aragones et al 2008, Schneider et al 2009). In line with our data, a single study assessing vessel density in the PHD1$^{-/-}$ brain, did not report any difference either (Chen et al 2012).

Rather, our data indicate that a cell-autonomous neuronal mechanism contributes to the increased resistance of the PHD1$^{-/-}$ brain against brain ischemia. Neuronal cultures lacking PHD1 were indeed protected against ischemia (i.e. deprivation of oxygen and nutrients) as well, indicating that PHD1 deficiency affects intrinsic neuronal properties that increase their ischemic resilience.

Our ongoing studies show that PHD1$^{-/-}$ neurons were not only protected against ischemia but also against glutamate excitotoxicity (data not shown). Excitotoxicity refers to the pathological overactivation of NMDA-receptors by glutamate. In ischemic conditions extracellular glutamate levels rise due to the rundown of ion gradients and ATP depletion (Hertz 2008). Excitotoxicity is one the main mechanisms contributing to neuronal ischemic death both *in vitro* and *in vivo* (Hertz 2008). Pilot experiments showed that the protection of PHD1-deficient neurons against excitotoxicity was not due to different levels or calcium-conducting properties of NMDA-receptors, nor due to altered expression of glutamate reuptake transporters. Also, we did not observe genotypic differences in expression levels of a variety of neurotrophin genes, which are known to increase neuronal survival in conditions of ischemia and excitotoxicity (Almeida et al 2005, Brandoli et al 1998, Ferenz et al 2012, Klumpp et al 2006).

3 METABOLIC REWIRING IN PHD1 DEFICIENT NEURONS

PHDs are considered as integrators and orchestrators of cellular metabolism. Most of the known metabolic effects of reduced PHD activity are HIF-dependent and aim to reduce oxygen consumption and energy expenditure (see also figure 5 in chapter I). In the context of ischemia, these HIF-mediated metabolic changes have been mainly considered as a means of oxygen-independent energy generation. Also in the brain and in cultured neurons, aspecific PHD inhibitors as well as PHD2 deletion were shown to induce the transcription of glycolytic genes (Baranova et al 2007, Kunze et al 2012, Zaman et al 1999). However, without demonstrating any functional or biological significance, these studies leave the link between PHDs and cellular metabolism in the brain largely enigmatic. Here, we uncovered a novel metabolic reprogramming in PHD1$^{-/-}$ neurons, as summarized in figure 21. Many novel insights and intriguing questions come forth from these findings as detailed in the next paragraphs.

3.1 PHD1 deficiency in neurons does not phenocopy the metabolic phenotype in the PHD1$^{-/-}$ muscle

First of all, these findings implicate that the metabolic features of PHD1$^{-/-}$ neurons do not mimic the metabolic phenotype in the ischemia tolerant PHD1$^{-/-}$ muscle (Figure 21). PHD1$^{-/-}$ muscle fibers showed a striking reduction in oxygen consumption in combination with an enhanced glycolytic flux and lactate production (Aragones et al 2008). Conversely, oxygen consumption was not affected in PHD1$^{-/-}$ neurons and, instead of an increase in glycolysis, they rather showed a modest reduction in their glycolytic flux. This finding was rather surprising given the overwhelming evidence that HIF-α stabilization promotes the glycolytic flux via transcriptional activation of several glycolytic genes (Schneider et al 2009). In contrast, a decrease in glycolysis was recently described in PHD3 deficient fibroblasts, which was attributed to the lack of hydroxylation and degradation of the glycolytic enzyme PKM2 (Luo et

al 2011). A possible direct interaction between PHD1 and PKM2 remains elusive. We also discovered that neuronal PHD1 deficiency increased the flux through the oxPPP. To the best of my knowledge, this is the first study to show a link between PHDs and the oxPPP. It is unclear at the moment whether this is a cell type-specific effect limited to neurons. Of note, in absolute terms the decrease in glucose flux through glycolysis is more pronounced than the increase in PPP flux.

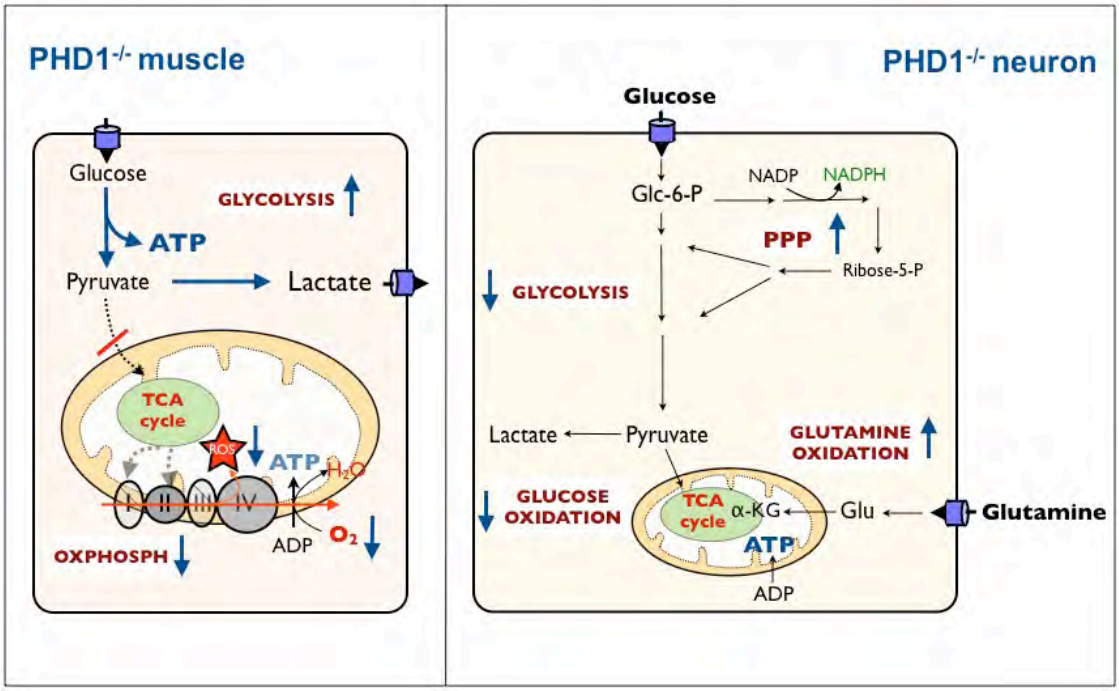

Figure 21: Comparison of metabolic rewiring in PHD1$^{-/-}$ muscle fibers versus PHD1$^{-/-}$ neurons.

The left panel shows a schematic overview of the metabolic changes in the PHD1$^{-/-}$ muscle fibers. Entry of pyruvate in the mitochondria is reduced. As a consequence, less oxygen is consumed and less ROS are generated in the mitochondria (OXPHOSPH: oxidative phosphorylation). Glycolytic flux and lactate excretion are increased. The right panel summarizes the metabolic alterations in the PHD1$^{-/-}$ neurons. Glucose is shifted away from glycolysis into the PPP. Due to reduced entry of glycolytic intermediates in the mitochondria, glucose oxidation is reduced. Oxygen consumption and ATP production are however similar due to the increased oxidation of glutamine. For reasons of simplicity and clarity, the stoichiometry of the reactions has been omitted.

A link between PHDs and glycogen metabolism has been suggested by the observation that hypoxia induces the expression of the enzymes responsible for glycogen degradation and synthesis (glycogen phosphorylase and glycogen synthase respectively) (Favaro et al 2012). Complementary studies in the host laboratory showed that loss of PHD1 increased glycogen accumulation in the liver. Nevertheless, the preliminary observation that PHD1$^{-/-}$ neurons show a higher glycogen content was rather unexpected, given the common belief that neurons cannot store glycogen (DiNuzzo et al 2011).

3.2 PHD1 deficiency has cell-type specific molecular mediators

Another interesting insight coming forth from these studies is that PHD1 deficiency, besides inducing different metabolic phenotypes, also has cell-type specific molecular mediators. In the PHD1$^{-/-}$ muscle, ischemia tolerance was at least partially attributed to HIF-2α (Aragones et al 2008). In PHD1$^{-/-}$ neurons however, HIF-1α levels were not affected and HIF-2α could not be detected. In addition, silencing of HIF-2α or HIF-1α failed to affect the ischemic protection in PHD1$^{-/-}$ neurons. A recent study did not observe HIF-1α accumulation in the PHD1$^{-/-}$ brain either (Chen et al 2012). Instead, pilot experiments showed that PHD1$^{-/-}$ neurons have enhanced NF-κB activity with increased protein levels of p65 and c-Rel, two members of the NF-κB transcription factor family (data not shown). HIF-independent targets of the PHDs are increasingly being recognized and probably, we know merely a small fraction of them. Interesting in this regard is that during evolution, PHDs arose earlier than HIFs (Place et al 2011). Nevertheless, in the brain, the literature on the protective effects of PHD inhibition is primarily HIF-1α focused (Nagel et al 2010, Quaegebeur and Carmeliet 2010). An intriguing finding is that our *in vitro* ischemia data might even point to an antagonistic role of HIF-1α, in line with an earlier study reporting a beneficial effect of HIF-1α silencing in an assay of neuronal oxidative death (Siddiq et al 2009). Still,

the *in vivo* significance of HIF-1α and HIF-2α in stroke needs further investigation.

4 How would enhanced oxPPP flux and glycogen accumulation in neurons protect against ischemia?

Based on our findings, we propose the following mechanisms to explain the neuroprotective effect of PHD1 loss (Figure 22):

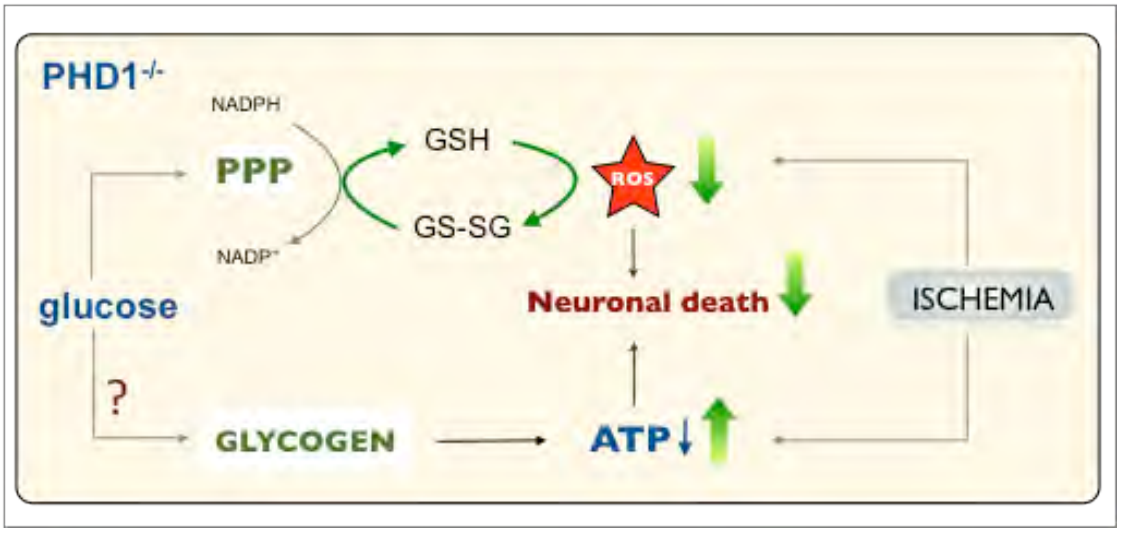

Figure 22: Model of preserved redox and energy homeostasis in the PHD1$^{-/-}$ neurons during ischemia

PHD1 deficiency in neurons shunts glucose from glycolysis into the PPP. Via increased NADPH generation, this will accelerate the glutathione recycling, improving redox homeostasis in conditions such as neuronal ischemia. The increased glycogen accumulation might additionally prevent an energetic crisis as an alternative and more efficient energy fuel for ATP generation during ischemia.

4.1 Enhanced oxPPP flux ensures redox homeostasis

In our study, we described for the first time that PHD1$^{-/-}$ neurons shunt more glucose through the oxPPP as compared to wild type neurons. In this way, they generate more of the reducing equivalent NADPH to replenish the pool of

reduced glutathione. This increase in oxPPP flux was associated with a decrease in glycolytic flux, raising the possibility that the regulation of this metabolic shift is at the level of the glycolysis/PPP crossroad. The increased oxPPP flux and consequently accelerated recycling of reduced glutathione enabled PHD1$^{-/-}$ neurons to detoxify H_2O_2 at a higher rate. Indeed, we observed an improved scavenging capacity of H_2O_2 in PHD1$^{-/-}$ neurons, which was negatively affected upon oxPPP blockade.

Given the widely held notion that oxidative stress in brain ischemia is heavily intertwined in the pathological cascade of events that leads to neuronal death (Chan 2001, Moskowitz et al 2010), the increase in PPP flux is likely to confer protection in this context. Some supporting evidence for this model exists in the literature, even though the role of the PPP in the brain remains understudied and even controversial. The NADPH molecules generated in the PPP appear to have opposing fates. As a reducing co-factor of glutathione reductase, NADPH will improve the anti-oxidant capacity, whereas as a cofactor of NADPH oxidase, it will promote superoxide generation (Stanton 2012). NADPH oxidase is a multi-subunit enzyme complex in the membrane that when activated generates superoxides by using NADPH as an electron donor (Bedard and Krause 2007). While this activity has been well described in activated neutrophils, its relevance in neurons is debated (Bedard and Krause 2007).

This intriguing conundrum of both ROS promoting and ROS reducing effects of the PPP is so far not well understood and probably explains the diverging reported results on the role of PPP in neurons. Flux through the oxPPP was found to be crucial for neuronal anti-oxidant defense, since PFKFB3 overexpression, which withdraws glucose from the PPP, resulted in oxidative death (Herrero-Mendez et al 2009). The same group also discovered that excitotoxicity impaired proteasomal degradation of PFKFB3, thereby reducing the PPP flux, whereas PFKFB3 silencing in these conditions was protective (Rodriguez-Rodriguez et al 2012). Conversely, other studies suggested that PPP powers NADPH oxidase activity in ischemia and

excitotoxicity (Brennan et al 2009, Suh et al 2008). On another note, the PPP flux could also fuel nucleotide synthesis via the generation of ribose-5-phosphate (Stanton 2012), which could facilitate DNA repair of the accumulating oxidative DNA damage.

The role of PPP in stroke specifically has been little studied. One study assessed the effect of silencing glucose-6-phosphate-dehydrogenase (G6PD, the rate-limiting enzyme of the PPP). This was found to reduce infarct size when silenced at the onset of stroke (probably by inhibition of NADPH oxidase activity) whereas at a later stage, inhibition of the PPP flux aggravated stroke outcome (Zhao et al 2012). Recently, levels of Tp53-Induced Glycolysis and Apoptosis regulator (TIGAR) were found to determine stroke size (Li et al 2014). TIGAR is a bisphosphatase that inhibits the activity of PFK-1 and thereby favors glucose utilization by the oxPPP flux at the expense of glycolysis. Therefore, the protective effect of TIGAR overexpression in stroke could possibly be attributed to an enhanced PPP flux. Still, since other functions of TIGAR are being increasingly recognized (Gerin et al 2014), the causality of this finding requires further research.

Notably, our data do not exclude that PHD1$^{-/-}$ neurons activate other pathways besides the oxPPP that might contribute to their improved ROS scavenging capacity. Possible candidates in this regard might be the mitochondrial NADPH generating enzymes such as malic enzyme, isocitrate dehydrogenase and nucleotide nicotinamide transhydrogenase (Arkblad et al 2002, Minich et al 2003, Vogel et al 1998) as well as the recently described folate-dependent NADPH production by methylenetetrahydrofolate dehydrogenase (Fan et al 2014, Lewis et al 2014). Another puzzling question is how the oxidative PPP flux in the cytosol can contribute to ROS scavenging in the mitochondria, putatively the main ROS generating site during ischemia. Different possible NADPH shuttles between the cytosol and the mitochondria can be put forward, yet, their contribution needs further investigation. Of interest, PFKFB3 inhibition is believed to protect against excitotoxicity via an increase of the PPP flux (Rodriguez-Rodriguez et al 2012).

4.2 COUNTERACTING THE HYPOXIC OXPPP → GLYCOLYSIS SHIFT

In most cells, hypoxia will via HIF-1α shift energy metabolism from mitochondrial respiration towards anaerobic glycolysis, which pursues oxygen-independent energy production. We showed that also neurons respond to hypoxia by promoting their glycolytic flux, in accordance with the existing literature (Malthankar-Phatak et al 2008). Many reasons can be put forward that would explain the potential benefit of these metabolic changes (reduction of mitochondrial ROS production, energy compensation by glycolytic ATP, etc.). Still, this study might provide a clue as to why neurons are so vulnerable to ischemia despite this metabolic adaptation. We observed that the increased glycolysis in hypoxic neurons was associated with a complete disappearance of the flux through the oxPPP. This finding corroborates the idea of a reciprocal regulation of the oxPPP/glycolysis balance and lets us speculate whether hypoxia would augment PFKFB3 protein levels that withdraw glucose from the PPP flux. Interestingly, both in cell culture and animal models of neurodegenerative disorders signs of increased glycolysis have been described as well (D'Alessandro et al 2011, Ferreira et al 2011, Yao et al 2009), raising the question whether also here a similar detrimental metabolic shift is taking place, depleting neurons from their vitally important PPP flux. PPP flux alterations in neurodegenerative disorders have not been studied so far.

PHD1 deficiency will not only promote oxPPP flux in baseline – normoxic – conditions. Our observation that PHD1$^{-/-}$ neurons maintain a residual PPP flux during hypoxia is likely to be even more relevant for their hypoxia tolerance, as at that time redox homeostasis will be acutely threatened. At the same moment, glycolysis levels in PHD1$^{-/-}$ neurons do not reach the same level as in their wild type counterparts. Taken together, these data indicate that the expected hypoxia-induced metabolic reprogramming is attenuated by PHD1 deficiency. This would implicate that loss of PHD1, by

counteracting the HIF-mediated metabolic changes, would enable redox control in hypoxic conditions.

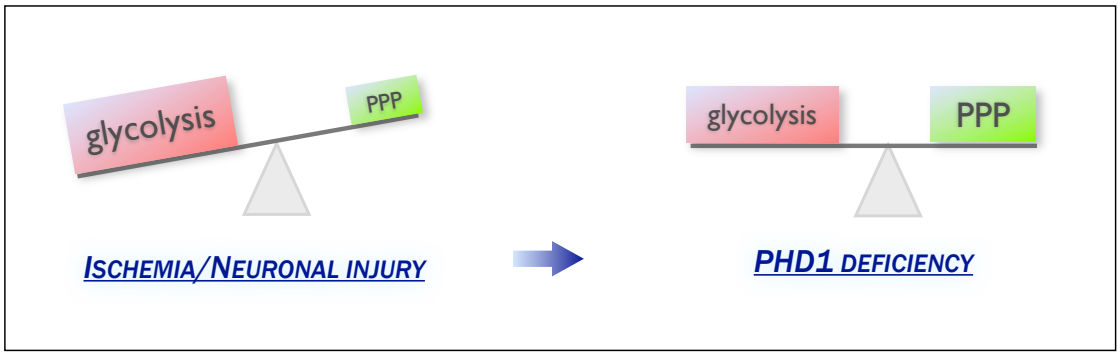

Figure 23: Model on effect of PHD1 deficiency on glycolysis/PPP balance in conditions of neuronal injury

Many conditions of neuronal injury such as excitotoxicity, ischemia and neurodegeneration are associated with an increased glycolysis. Excitotoxicity was previously shown to reduce PPP, and in this study we show that hypoxia nullifies the flux through the PPP. This metabolic shift from PPP towards glycolysis is likely to adversely affect neuronal survival, as these changes will deplete neurons from their major anti-oxidant mechanism. We postulate that PHD1 deficiency enacts a neuroprotective effect by shunting glucose back from glycolysis into the PPP, thereby restoring the balance between glycolysis and the oxPPP.

4.3 GLYCOGEN STORES AS AN ALTERNATIVE ENERGY SOURCE POTENTIALLY CONTRIBUTE TO ENERGY HOMEOSTASIS

Our preliminary observation that PHD1$^{-/-}$ neurons stored more glycogen was rather surprising given the existing view on glycogen in the brain. Glycogen storage in the brain has been assumed to be an astrocyte-specific process (Brown and Ransom 2014). The common belief is that neurons are not able to store glycogen and that neuronal glycogen accumulation might even be deleterious (DiNuzzo et al 2011). It was only very recently that a small pool of glycogen in neurons was recognized to be important for energy generation in ischemic conditions (Saez et al 2014). Although increased glycogen content in

PHD1$^{-/-}$ neurons could readily explain their preserved energy charge during prolonged oxygen nutrient starvation *in vitro*, its relative contribution and *in vivo* relevance will have to be demonstrated. Of note, in contrast to the ATP consuming process of glucose uptake, degradation of glycogen into glycolytic intermediates is energetically more favorable and might therefore be provide an additional energy-saving advantage in hypoxia.

4.4 RELEVANCE OF ENHANCED GLUTAMINE OXIDATION?

In baseline conditions (i.e normoxia), the increased ATP production through the enhanced glutamine oxidation likely compensates for the decrease in ATP production from the reduction in glucose oxidation. It is however less clear whether the increased preference for glutamine as oxidative substrate would contribute to the ischemic protection. From an energetic standpoint, low oxygen availability in ischemic conditions would limit oxidative metabolism and hence prevent glutamine's contribution to energy production. However, oxygen tension in the mitochondria will only become limiting in nearly anoxic conditions (Semenza 2012). Still, the role of glutamine in brain ischemia remains obscure, which likely relates to the different fates of glutamine in neurons: besides energy purposes through mitochondrial oxidation, glutamine also serves as precursor for the neurotransmitter glutamate and as precursor for the anti-oxidant glutathione (McKenna 2007). There are reports showing that the presence of glutamine in the medium in conditions of mitochondrial dysfunction even aggravates neuronal cell death (Goldberg et al 1988, Stelmashook et al 2007, Stelmashook et al 2010). This is likely due to enhanced production of glutamate, facilitating excitotoxicity.

5 IS PHD1 INHIBITION ALSO PROTECTIVE IN NEURODEGENERATIVE DISORDERS?

Acute ischemic stroke might be the most striking example of acute deregulated oxygen homeostasis. Still, many other brain diseases are also characterized by metabolic and vascular deficits (Quaegebeur and Carmeliet 2010, Quaegebeur et al 2011). Amyotrophic lateral sclerosis is a neurodegenerative disorder characterized by the progressive dysfunction and death of motorneurons resulting in paralysis, muscle atrophy and ultimately death of the patient (Van Damme and Robberecht 2014). Many pathogenetic mechanisms have been implicated in this heterogeneous disease among which hypoxic and oxidative stress, rationalizing the study of PHD1 deficiency in this disorder (Quaegebeur and Carmeliet 2010). We discovered a beneficial effect of genetic PHD1 knockdown in the $SOD1^{G93A}$ model on survival and onset of paralysis. Whether metabolic alterations in motorneurons are responsible for attenuating motor neuron death requires further research. Whether PHD1 deficiency would be beneficial in other neurodegenerative disease such as Alzheimer's and Parkinson's disease remains an outstanding question as well.

6 IS PHD1 INHIBITION A VALID THERAPEUTIC APPROACH IN STROKE?

We showed that silencing of PHD1 in the brain via intracerebroventricular delivery of antisense oligonucleotides protects against brain ischemia. This study is to the best of my knowledge the first study where pharmacological inhibition of a specific PHD isoform in stroke was investigated. The existing PHD inhibitors with proven benefit in experimental stroke studies carry major disadvantages for their clinical translation. Their lack of specificity for PHD proteins gives rise to an unfavorable side-effect profile preventing them to become a valid reagent for assessment in clinical trials (Fraisl et al 2009). Therefore, our therapeutic success with PHD1 inhibition reflects an important

proof of concept in the therapeutic assessment of PHD inhibitors in stroke and rationalizes a continued search for PHD-isoform specific inhibitors.

In this study, silencing of PHD1 was initiated prior to stroke induction. This implicates PHD1 inhibition as a preconditioning strategy. In cerebral preconditioning, the brain is exposed to a controlled stressful stimulus, such as transient ischemia, to an extent that neuronal function is slightly impaired, yet not irreversibly damaged. It has been extensively described that this will elicit a protective response, attenuating brain damage following a more severe stressor (Quaegebeur and Carmeliet 2010). PHD1 inhibition might be one of the underlying molecular mechanisms of cerebral preconditioning: by installing metabolic changes, PHD1 deficiency would prepare the brain in advance to cope with a future drop in oxygen supply. In that respect, PHD1 inhibition would be clinically useful in situations where the risk of cerebral ischemia is considered high such as upon a critical arterial stenosis (e.g. high grade carotid stenose, intracranial atherosclerosis, Moyamoya disease) or following a subarachnoid hemorrhage.

What about using PHD1 inhibition as a neuroprotective strategy after stroke? Beneficial effects within the neuroprotective window would implicate that PHD1 inhibition is not only able to prime brain tissue for ischemia, but also able to interfere with the ongoing series of detrimental events in the ischemic cascade. Based on different studies in humans, the therapeutic time window is likely to be rather short (within hours after stroke onset) (Tymianski 2013). Yet, in hemorrhagic stroke where via expansion of the hematoma the tissue might suffer from secondary ischemia, the therapeutic window might be longer. Some studies have reported beneficial effects with post-stroke administration of PHD inhibitors (Nagel et al 2011, Ogle et al 2012, Reischl et al 2014). Whether PHD1 inhibition will have a clinically relevant therapeutic time window will need further investigation.

As a final critical note, one could wonder why PHD1 inhibition would be effective as a stroke treatment whereas so far, neuroprotective drugs targeting oxygen radicals and excitotoxicity have repeatedly failed in clinical

trials (Ginsberg 2008). Many reasons can be put forward to explain this translational failure both at the clinical and the preclinical level. It has become clear that the ischemic cascade involves a complex interplay between deleterious processes, so that targeting one mechanism such as oxygen radicals or excitotoxicity is unlikely to have major effects (Ginsberg 2009). In addition, many stroke treatments have neglected the delicate and complex biology of oxygen radicals and NMDA-receptor signaling. Even though excesses of these are toxic, they both fulfill essential physiological signaling functions in neurons (Moskowitz et al 2010). Therapeutics should therefore be focused on attenuating the excessive effects rather than simply blocking either of them. PHD1 inhibition is in this regard an interesting approach since it appeals to an endogenous protective mechanism with pleiotropic actions. As for the clinical trials, without going into detail on their methodological issues that likely contributed to the translational difficulties, it will be crucial for strategies such as PHD1 inhibition to select an appropriate patient population and to hold on to a strict time window (Moskowitz et al 2013).

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Our study revealed that PHD1 is a valid therapeutic target in stroke and extended our insights into the role of PHD1 as a metabolic regulator. Neurons lacking PHD1 shift their glucose from glycolysis towards the PPP, thereby enhancing their anti-oxidant potential. Initial data also point to increased glycogen accumulation providing an alternative energy source during ischemia. Whether this metabolic rewiring in neuronal cultures is relevant for the protection against ischemia *in vivo* is the topic of further research. Additionally, this study represents a proof of concept that pharmacological inhibition of a specific PHD isoform governs neuroprotection in ischemic stroke.

Chapter VI

SUMMARY

The brain relies on a continuous supply of oxygen and nutrients. In ischemic stroke, an abrupt arterial occlusion results in oxygen and nutrient deprivation within the perfusion area of the affected vessel. As the second leading cause of death and the most common reason for severe disability, the medical, social and economic impact of stroke can hardly be overstated.

Below a certain threshold of blood flow reduction, neurons will undergo necrotic death due to complete bioenergetic failure. Surrounding this irreversibly lost tissue, there is a region that suffers moderate blood flow reduction. This ischemic penumbra is still viable and hence salvageable during a certain time window. A cascade of deleterious events will disrupt energy and redox homeostasis and result in ischemic death unless perfusion is restored via recanalization or if a neuroprotective drug interferes with this downward spiral.

Given the vital importance of maintaining oxygen and metabolic homeostasis, mammalian organisms are equipped with oxygen sensing mechanisms among which the Prolyl Hydroxylase Domain proteins (PHDs). PHDs are a class of oxygen sensors inducing adaptive processes to restore oxygen homeostasis in an oxygen-dependent manner. In hypoxia, PHDs lose their hydroxylation activity and thus activate a major transcriptional pathway governed by the hypoxia-inducible factors (HIFs). There are 3 different PHD isoforms (PHD1,2,3) and only during recent years their non-redundant and tissue-specific functions are being unraveled. The host lab previously documented that absence of PHD1 provided ischemia tolerance to muscle and liver cells through a metabolic reprogramming that switches from mitochondrial respiration to anaerobic glycolysis.

Aspecific PHD inhibitors were shown to be neuroprotective in acute

neurotoxic settings such as stroke, but their lack in specificity prevents translation into the clinical setting and any insight into the underlying mechanisms. These aspecific inhibitors have been linked to an increase in glycolysis, but whether this is responsible for the neuroprotective effect is unresolved. Remarkably, the precise role of metabolic alterations in the pathogenesis of neuronal injury as well as their significance in neuroprotection remains largely obscure. This doctoral work aimed to characterize the role of PHDs in ischemic stroke and to better understand the underlying molecular and cellular underpinnings of their effects.

We showed that deletion of oxygen sensor PHD1 largely prevented brain ischemia after ligation of the middle cerebral artery. We uncovered a novel metabolic rewiring in PHD1 deficient neurons that was strikingly different from the metabolic phenotype in the PHD1 deficient muscle. Neurons lacking PHD1 shunted more glucose into the oxidative pentose-phosphate pathway (oxPPP). The oxPPP represents an alternative route for glucose besides glycolysis, critical for redox homeostasis by generating NADPH, which is necessary to replenish reduced glutathione. In line with these findings, the capacity to scavenge oxygen radicals was improved in neurons lacking PHD1. Contrary to wild type neurons, PHD1 deficiency also enabled a residual flux through the oxPPP during hypoxia. In this way, PHD1 deficient neurons have an increased tolerability towards the surge in oxygen radicals during ischemia. Finally, silencing of PHD1 by intracerebroventricular delivery of antisense oligonucleotides was protective against brain ischemia. These findings not only identify a novel therapeutic target for ischemic stroke but also provide novel insights into the link between oxygen sensors, metabolism and neuroprotection.

SAMENVATTING

Hersenen hebben nood aan een continue toevoer van zuurstof en voedingsstoffen. Bij een beroerte zal een acute slagaderverstopping leiden tot een tekort aan zuurstof en voedingsstoffen in het gebied dat het verstopt bloedvat voorziet. Beroertes zijn momenteel de tweede doodsoorzaak en de belangrijkste oorzaak van zorgbehoevendheid. De medische, sociale en economische impact van beroertes kan dus moeilijk overschat worden.

Wanneer de bloedtoevoer onder een bepaalde drempel zakt, zal dit onmiddellijk tot necrose van neuronen leiden door een volledig energetisch falen. Rond deze kern van onomkeerbaar verloren weefsel is er een regio waar de bloedvoorziening slechts matig gedaald is. Omdat deze “ischemische penumbra” nog gedurende een bepaald tijdsvenster viabel is, kan deze nog gered worden. In dit gebied zal een reeks van schadelijke gebeurtenissen de energie en redox homeostase verstoren en uiteindelijk aanleiding geven tot celdood, tenzij dat recanalisatie de bloeddoorstroming herstelt of dat een neuroprotectief medicijn interfereert met deze neerwaartse spiraal.

Aangezien het onderhouden van een zuurstof- en metabool evenwicht van crucial belang is, zijn zoogdieren uitgerust met zuurstofsensor mechanismen waaronder de Prolyl Hydroxylase Domein proteïnen (PHDs). PHDs zijn een klasse van zuurstofsensoren die op een zuurstof-afhankelijke manier aanpassingsmechanismen activeren om het zuurstofevenwicht te herstellen. In hypoxie zullen PHDs hun hydroxylatie activiteit verliezen en zo een belangrijk transcriptie-programma activeren via de hypoxie-induceerbare factoren (HIFs). Er zijn drie verschillende PHD isovormen (PHD1, 2,3) en het is enkel gedurende de laatste jaren dat we hun niet-overlappende en weefsel-specifieke functies leren kennen. Het gastlaboratorium heeft in het verleden aangetoond dat de afwezigheid van PHD1 de spier en de lever resistent maakte aan ischemie en dit door een metabole reprogrammering waarbij mitochondriale respiratie werd ingeruild voor glycolyse.

Experimenteel onderzoek toonde reeds aan dat aspecifieke PHD inhibitoren neuroprotectief werken in acute neurotoxische settings zoals beroertes. Hun gebrek aan specificiteit bemoeilijkt echter de vertaling naar een klinische context en levert ook geen enkel inzicht in het onderliggend mechanisme. Deze aspecifieke inhibitoren werden geassocieerd aan een toegenomen glycolyse, maar het blijft onbekend in hoeverre dit verantwoordelijk is voor het neuroprotectief effect. Opmerkelijk is dat de precieze rol van metabole veranderingen in het ontstaan van neuronale schade alsook hun bijdrage in neuroprotectie grotendeels ongekend blijft. Deze doctoraatsthesis beoogde om de rol van de PHDs in beroertes te karakteriseren en om de onderliggende moleculaire en cellulaire mechanismen van hun effecten beter te begrijpen.

We hebben aangetoond dat deletie van de zuurstofsensor PHD1 grotendeels beschermt tegen hersenischemie na het afbinden van de middelste cerebrale arterie. We ontdekten een ongekende metabole reprogrammering in neuronen zonder PHD1 die opmerkelijk verschillend was van de metabole veranderingen in de PHD1-deficiënte spier. Neuronen zonder PHD1 stuurden meer glucose in de oxidatieve pentose fosfaat cascade. Via deze alternatieve route voor glucose wordt er NADPH aangemaakt dat noodzakelijk is voor het regenereren van gereduceerd glutathion, een belangrijk anti-oxidant in neuronen. In de lijn van deze bevindingen was de capaciteit om zuurstofradicalen te neutraliseren ook hoger in neuronen zonder PHD1. In tegenstelling tot wild type neuronen zorgde de afwezigheid van PHD1 voor een residuele flux door de pentose fosfaat cascade tijdens hypoxie. Op deze manier zijn PHD1-deficiënte neuronen beschermd tegen de stijging van zuurstofradicalen tijdens ischemie. Tenslotte, het verminderen van PHD1 door intracerebroventriculaire toediening van oligonucleotides beschermde ook tegen hersenischemie. Deze bevindingen identificeren niet enkel een nieuw therapeutisch doelwit voor ischemische beroertes, maar verschaffen ook nieuwe inzichten in de link tussen zuurstofsensoren, metabolisme en neuroprotectie.

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CURRICULUM VITAE

Name: Annelies Quaegebeur

Place and Date of Birth: Leuven, Belgium – April 13, 1984

EDUCATION/TRAINING

Institution	Degree	Year
Secondary school (Paridaens, Leuven)		2001
Medicine (K.U.Leuven)		
Bachelor Degree	*'Summa cum laude with congratulations of the jury'*	2004
Doctor in Medicine (M.D.)	*'Summa cum laude with congratulations of the jury'*	2008

PROFESSIONAL EXPERIENCES AND EMPLOYEMENT

2008 Master Thesis at laboratory 'Experimental Neurology', K.U.Leuven
Title: '*Role of ELP3 in motor neuron biology and disease'.*
Promotor*:* Prof. Dr. W. Robberecht

2008 – … Resident at department of Neurology, UZ Leuven

2009 – 2014 Ph.D. student at Vesalius Research Center, K.U.Leuven
Promotor: Prof. Dr. P. Carmeliet; Co-promotor: Prof. Dr. W. Robberecht

PUBLICATIONS

- Maes H*, Kuchnio A*, Peric A, Moens S, Nijs K, De Bock K, Quaegebeur A et al. Tumor vessel normalization by chloroquine independent of autophagy. Cancer Cell. 2014 Aug 11;26(2):190-206

- Schoors S*, Cantelmo AR*, Georgiadou M, Stapor P, Wang X, Quaegebeur A et al. Incomplete and transitory decrease of glycolysis: a new paradigm for anti-angiogenic therapy? Cell Cycle 2014 Jan 1;13(1):16-22

- Schoors S*, De Bock K*, Cantelmo AR*, Georgiadou M, Ghesquière B, Cauwenberghs S, Kuchnio A, Wong BW, Quaegebeur A et al. Partial and transient reduction of glycolysis by PFKFB3-blockade reduced pathological ocular angiogensis. Cell Metabolism 2014 Jan 7;19(1):37-48

- De Bock K*, Georgiadou M*, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, Quaegebeur A et al. Role of PFKFB3-driven glycolysis in vessel sprouting. Cell 2013 Aug1;154(3):651-6

- Storkebaum E, Quaegebeur A, Vikkula M, Carmeliet P. Cerebrovascular disorders: molecular insights and therapeutic opportunities. Nat Neurosc. 2011 Oct 26;14(11):1390-7

- Quaegebeur A, Lange C, Carmeliet P. The neurovascular link in health and disease: molecular mechanisms and therapeutic implications. Neuron 2011 Aug 11;71(3):406-424.

- Quaegebeur A, Segura I, Carmeliet P. Pericytes: blood-brain barrier safeguards against neurodegeneration? Neuron 2010 Nov 68:312-3.

- Quaegebeur A, Carmeliet P. Oxygen sensing: a common crossroad in cancer and neurodegeneration. Curr Top Microbiol Immunol. 2010;345:71-103.

Articles in revision & preparation

- Quaegebeur A et al. Deletion and inhibition of the oxygen sensor PHD1 protects against ischemic stroke via rewiring of neuronal metabolism. Manuscript in revision at Cell Metabolism.

- Quaegebeur A et al. PHD1 deficiency attenuates neurodegeneration in an ALS mouse model. Manuscript in preparation.

PRESENTATIONS

Selected abstracts and short talks in national and international meetings

- *Oral presentation* at VIB PhD symposium (Blankenberge, Belgium, February 2013). Oxygen sensors, metabolism and neuroprotection: unexpected liaisons with therapeutic opportunities for neurodegeneration?
- *Oral presentation* at LIND seminar (Mechelen, Belgium, December 2012). Oxygen sensors, neuroprotection and metabolism: unexpected liaisons and therapeutic opportunities for neurodegeneration?
- *Poster presentation* at Cell-VIB: Angiogenesis, Metabolic regulation and Cancer Biology (Leuven, Belgium, July 2012). Oxygen sensors, neuroprotection and metabolism: unexpected liaisons and therapeutic opportunities for neurodegeneration?
- *Poster presentation* at 10th Meeting of International Conference of Brain Energy Metabolism (Monterey, California, US, April 2012). Oxygen sensors, neuroprotection and metabolism: unexpected liaisons and therapeutic opportunities for neurodegeneration.
- *Oral presentation* at VRC Mini-Symposium (Vesalius Research Center, Leuven, Belgium, October 2011). Oxygen sensors, metabolism and neuroprotection: unexpected liaisons with therapeutic opportunities for neurodegeneration?
- *Poster Presentation* at the 19th Meeting of the European Neurological Society (Milan, Italy, June 2009) Occipital lobe epilepsy, myoclonus, refractory status epilepticus, ataxia and polyneuropathy represent the core features of a syndrome caused by POLG1 mutations.

Invited lectures

- Quaegebeur A. Oxygen sensors, neuroprotection and metabolism: unexpected liaisons and therapeutic opportunities for neurodegeneration? Eibsee meeting (Münich, Germany, November 2012)
- Quaegebeur A. Oxygen sensors, neuroprotection and metabolism: unexpected liaisons and therapeutic opportunities for neurodegeneration? Lecture at Department of Molecular and Developmental Genetics (VIB, K.U.Leuven, Belgium, September 2012)

- Quaegebeur A & Carmeliet P. Bloedvaten en stofwisseling: waarom zuurstof belangrijk is in ALS. ALS liga symposium (UZ Leuven, Belgium, October 2010)

AWARDS & FELLOWSHIPS

- FWO PhD fellowship 2009 - 2013
- Junior Investigator International Travel Award for the 10th International Conference for Brain Energy Metabolism (April 2012)
- Fonds "A Cure for ALS". PHD1 as novel neuroprotective target in ALS (December 2012)